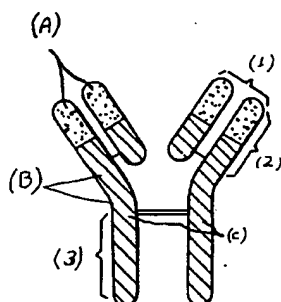


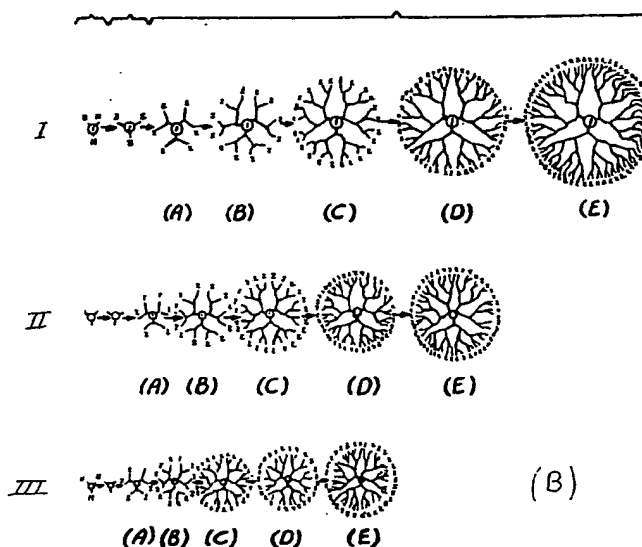


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(54) Title: STARBURST CONJUGATES

(A)



(B)

(57) Abstract

Starburst conjugates which are composed of at least one starburst polymer in association with at least one unit of a carried pharmaceutical material have been prepared. These conjugates have particularly advantageous properties due to the unique characteristics of the starburst polymer.

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STARBURST CONJUGATES

The present invention concerns the use of dense star polymers as carriers for pharmaceutical materials. In recent years polymers referred to as dense star
5 polymers or starburst polymers have been developed. It has been found that the size, shape and properties of these dense star polymers or starburst polymers can be molecularly tailored to meet specialized end uses. Starburst polymers have significant advantages which
10 can provide a means for the delivery of high concentrations of carried material per unit of polymer, controlled delivery, targeted delivery and/or multiple species delivery or use.

15 In its broadest aspect, the present invention is directed to polymer conjugate materials comprising dense star polymers or starburst polymers associated with desired materials (hereinafter these polymer
20 conjugates will frequently be referred to as "starburst conjugates" or "conjugates"), process for preparing these conjugates, compositions containing the

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conjugates, and methods of using the conjugates and compositions.

5 The conjugates of the present invention are suitable for use in a variety of applications where specific delivery is desired, and are particularly suited for the delivery of biologically active agents. In a preferred embodiment of the present invention, the starburst conjugates are comprised of one or more
10 starburst polymers associated with one or more bioactive agents.

The starburst conjugates offer significant benefits over other carriers known in the art due to
15 the advantageous properties of the starburst polymers. Starburst polymers exhibit molecular architecture characterized by regular dendritic branching with radial symmetry. These radially symmetrical molecules are referred to as possessing "starburst topology".
20 These polymers are made in a manner which can provide concentric dendritic tiers around an initiator core. The starburst topology is achieved by the ordered assembly of organic repeating units in concentric,
25 dendritic tiers around an initiator core; this is accomplished by introducing multiplicity and self-replication (within each tier) in a geometrically progressive fashion through a number of molecular generations. The resulting highly functionalized
30 molecules have been termed "dendrimers" in deference to their branched (tree-like) structure as well as their oligomeric nature. Thus, the terms starburst oligomer and starburst dendrimer are encompassed within the term starburst polymer. Topological polymers, with size and
35 shape controlled domains, are dendrimers that are covalently bridged through their reactive terminal

-3-

groups, which are referred to as starburst "bridged dendrimers." The term bridged dendrimer is also encompassed within the term "starburst polymer".

5 The following description of the figures aid in understanding the present invention.

Figure 1 depicts various generations of starburst dendrimers..

10 Figure 2A depicts a dendrimer having unsymmetrical (unequal) branch junctures.

Figure 2B depicts a dendrimer having symmetrical (equal) branch junctures.

15 Figure 3 depicts dendrimer sizes relative to antibody dimensions.

Figure 4 shows carbon-13 spin lattice relaxation times (T₁) for aspirin incorporated into various dendrimer generations. (Example 1)

Figure 5 shows the results of the dynamic analysis of Example 2.

25 Figure 6 shows the influence of generation 6.5 dendrimer on the dialysis rate of pseudoephedrine at pH 9.5 from Example 2.

30 Figure 7 shows the effect of dendrimer hydrolysis on the permeability of pseudoephedrine of Example 3.

Example 8 Comparison of Percent Salicylic Acid released into the receptor compartment in the presence of starburst polymer (Gen = 4.0) at pH 5.0 and 6.65 with salicylic acid control, Example 4.

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Figure 9 Comparison of percent salicyclic acid lost from donor compartment with starburst polymer (Gen = 4.0) in receptor compartment at pH 8.0 to salicyclic acid content, Example 4.

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Figure 10 Comparison of percent salicyclic acid lost from donor compartment in presence of starburst polymer (Gen =4.5) to salicyclic acid control, Example 4.

10

The starburst polymers are illustrated by Figure 1 wherein (I) represents an initiator core (in this figure a tri-functional initiator core shown by the far left drawing,); Z represents a terminal group, shown in the first instance by the second drawing from the left, referred to as a starbranched oligomer; A, B, C, D, and E represent particular molecular generations of starburst dendrimers; and $(A)_n$, $(B)_n$, $(C)_n$, $(D)_n$, and $(E)_n$ represent starburst bridged dendrimers.

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The starburst dendrimers are unimolecular assemblages that possess three distinguishing architectural features, namely, (a) an initiator core, (b) interior layers (generations, G) composed of repeating units, radially attached to the initiator core, and (c) an exterior surface of terminal functionality (i.e., terminal functional groups)

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attached to the outermost generation. The size and shape of the starburst dendrimer molecule and the functional groups present in the dendrimer molecule can be controlled by the choice of the initiator core, the number of generations (i.e., tiers) employed in creating the dendrimer, and the choice of the repeating units employed at each generation. Since the dendrimers can be readily isolated at any particular

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generation, a means is provided for obtaining dendrimers having desired properties.

The choice of the starburst dendrimer components affects the properties of the dendrimers. The initiator core type can affect the dendrimer shape, producing (depending on the choice of initiator core), for example, spheroid-shaped dendrimers, cylindrical or rod-shaped dendrimers, ellipsoid-shaped dendrimers, or mushroom-shaped dendrimers. Sequential building of generations (i.e., generation number and the size and nature of the repeating units) determines the dimensions of the dendrimers and the nature of their interior.

Because starburst dendrimers are branched polymers containing dendritic branches having functional groups distributed on the periphery of the branches, they can be prepared with a variety of properties. For example, the macromolecules depicted in Figure 2A, and the starburst dendrimers, such as those depicted in Figure 2B can have distinct properties due to the branch length. The dendrimer type shown in Figure 2A possesses unsymmetrical (unequal segment) branch junctures, exterior (i.e., surface) groups (represented by Z'), interior moieties (represented by Z) but much less internal void space. The preferred dendrimer type shown in Figure 2B possesses symmetrical (equal segment) branch junctures with surface groups (represented by Z'), two different interior moieties (represented respectively by X and Z) with interior void space which varies as a function of the generation (G). The dendrimers such as those depicted in Figure 2B can be advanced through enough generations to totally enclose and contain void space,

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to give an entity with a predominantly hollow interior and a highly congested surface. Also, starburst dendrimers, when advanced through sufficient generations exhibit "starburst dense packing" where the surface of the dendrimer contains sufficient terminal moieties such that the dendrimer surface becomes congested and encloses void spaces within the interior of the dendrimer. This congestion can provide a molecular level barrier which can be used to control diffusion of materials into or out of the interior of the dendrimer.

Surface chemistry of the dendrimers can be controlled in a predetermined fashion by selecting a repeating unit which contains the desired chemical functionality or by chemically modifying all or a portion of the surface functionalities to create new surface functionalities. These surfaces may either be targeted toward specific sites or made to resist uptake by particular organs or cells e.g. by reticuloendothelial cells.

In an alternative use of the starburst dendrimers, the dendrimers can themselves be linked together to create polydendric moieties (starburst "bridged dendrimers") which are also suitable as carriers.

In addition, the dendrimers can be prepared so as to have deviations from uniform branching in particular generations, thus providing a means of adding discontinuities (i.e., deviations from uniform branching at particular locations within the dendrimer) and different properties to the dendrimer.

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The starburst polymers employed in the starburst conjugates of the present invention can be prepared according to methods known in the art, for example, U. S. Patent 4,587,329.

5

Dendrimers can be prepared having highly uniform size and shape and most importantly allow for a greater number of functional groups per unit of surface area of the dendrimer, and can have a greater number of functional groups per unit of molecular volume as compared to other polymers which have the same molecular weight, same core and monomeric components and same number of core branches as the starburst polymers. The increased functional group density of the dense starburst polymers may allow a greater quantity of material to be carried per dendrimer. Since the number of functional groups on the dendrimers can be controlled on the surface and within the interior, it also provides a means for controlling the amount of bioactive agent to be delivered per dendrimer. In a particularly preferred embodiment of the present invention the starburst polymers, particularly the starburst dendrimers, are targeted carriers of bioactive agents capable of delivering the bioactive agents to a particular target organism or to a particular determinant or locus in a target organism.

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An analogy can be made between early generation starburst dendrimers (i.e. generation = 1-7) and classical spherical micelles. The dendrimer-micelles analogy was derived by comparing features which they had in common such as shape, size and surface.

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Table I

5	<u>Parameter</u>	<u>Regular Classical Micelles</u>	<u>Starburst Dendrimers</u>
	Shape	Spherical	Spherical
	Size (diameter)	20-60Å	17-67Å
10	Surface aggregation number	4-202	Z=6-192 (Z is the number of surface groups)(generation = 2-7)
	area/surface group (Å ²)	130-80Å ²	127-75Å ²
	(1Å = 10 ⁻¹ nm; 1Å ² = 10 ⁻² nm ²)		

15

In Table I, the shape was verified by scanning transmission electron micrographs (STEM) microscopy and intrinsic viscosity (η) measurements. The size was verified by intrinsic viscosity $[\eta]$ and size exclusion chromatography (SEC) measurements. The surface aggregation numbers were verified by titrimetry and high field NMR. The area/surface group was calculated from SEC hydrodynamic measurements.

25

The first five generations of starburst polyamidoamine (PAMAM) dendrimers are microdomains which very closely mimic classical spherical micelles in nearly every respect (i.e. shape, size, number of surface groups, and area/surface groups). A major difference, however, is that they are covalently fixed and robust compared to the dynamic equilibrating nature of micelles. This difference is a significant advantage when using these microdomains as encapsulation devices.

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-9-

As further concentric generations are added beyond five, congestion of the surface occurs. This congestion can lead to increased barrier characteristics at the surface and manifests itself as a smaller surface area per head (surface) group as shown in Table II.

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Table II
PAMAM Dendrimer Features vs. Generation

Generations	1	2	3	4	5	6	7	8	9
# of surface groups, Z	3	6	12	24	48	96	192	384	768
Molecular wt.	275	875	2411	5147	10,619	21,563	43,541	87,227	174,779
Diameter* measured SEC	10.4Å	15.8Å	22Å	31Å	40Å	53Å	67Å	76Å	88Å
Surface area per dendrimer	366Å ²	783Å ²	1519Å ²	3018Å ²	5024Å ²	8,820Å ²	14,096Å ²	18,136Å ²	36,083Å ²
Surface area per Z group	122Å ²	131Å ²	127Å ²	126Å ²	104Å ²	92Å ²	73Å ²	47Å ²	32Å ²
Distance between Z groups	12.4Å	12.8Å	12.7Å	12.6Å	11.5Å	10.8Å	9.8Å	7.75Å	6.28Å
Void Volume	311.6Å ³	1,470.2Å ³	4,737.9Å ³	11,427.0Å ³	---	---	---	---	---

* Hydrodynamic diameters determined by size exclusion chromatography measurements calibrated against monodisperse ($M_w = 1.02$) polyethyleneoxide standards.

$1\text{Å} = 10^{-1}\text{ nm}$; $1\text{Å}^2 = 10^{-2}\text{ nm}^2$; $1\text{Å}^3 = 10^{-3}\text{ nm}^3$.

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For example, amine terminated generations 5.0, 6.0, 7.0, 8.0 and 9.0 have decreased surface areas of 104, 92, 73, 47 and 32\AA^2 per Z group, respectively. This characteristic corresponds to a transition from a
5 less congested micelle-like surface to a more congested bi-layer/monolayer barrier-like surface normally associated with vesicles (liposomes) or Langmuir-Blodgett type membranes.

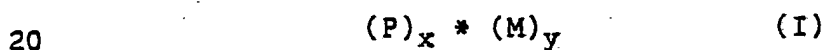
10 If this surface congestion is occurring, the change in physical characteristics and morphology should be observed as the generations increase from the intermediate generation (6-8) to the more advanced
15 generations (9 or 10). The scanning transmission electron micrographs (STEM) for generations = 7.0, 8.0 and 9.0 were obtained after removing the methanol solvent from each of the samples to provide colorless,
20 light yellow solid films and staining with osmium tetroxide. The morphological change predicted occurred at the generation G=9.0 stage. The microdomains at generation = 9.0 measure about 33\AA in diameter and are surrounded by a colorless rim which is
25 about 25\AA thick. Apparently methanolic solvent has been entrapped within the 25\AA outer membrane-like barrier to provide the dark stained interior. Thus, at generation = 9.0, the starburst PAMAM is behaving
30 topologically like a vesicle (liposome). However, this starburst is an order of magnitude smaller and very monodispersed compared to a liposome. Consequently, the present dendrimers can be used to molecularly encapsulate solvent filled void spaces of as much
35 diameter as about 33\AA (volume about $18,000\text{\AA}^3$) or more. These micelle sized prototypes appear to behave like a covalently fixed liposome in this advanced generation

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stage. This behavior enables these prototypes to serve as drug delivery agents or as carriers for non-chelating radionuclides in starburst antibody conjugates for the treatment of various mammalian diseases.

Dendrimers suitable for use in the conjugates of the present invention include the dense star polymers or starburst polymers described in U. S. Patents 4,507,466, 4,558,120, 4,568,737 and 4,587,329.

In particular, the present invention concerns a starburst conjugate which comprises at least one starburst polymer associated with at least one carried pharmaceutical material. Starburst conjugates included within the scope of the present invention include those represented by the formula:



wherein each P represents a dendrimer;

x represents an integer of 1 or greater;

each M represents a unit (for example, a molecule, atom, ion, and/or other basic unit) of a carried pharmaceutical material, said carried pharmaceutical material can be the same carried pharmaceutical material or a different carried pharmaceutical material, preferably the carried pharmaceutical material is a bioactive agent;

y represents an integer of 1 or greater; and

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* indicates that the carried pharmaceutical material is associated with the dendrimer.

5 Preferred starburst conjugates of formula (I) are those in which M is a drug, radionuclide, chelant, chelated metal, toxin, antibody, antibody fragment, antigen, signal generator, signal reflector, or signal absorber; particularly preferred are those in which $x=1$, and $y=2$ or more.

10 Also included are starburst conjugates of formula (I) wherein the starburst dendrimers are covalently linked together, starburst bridged dendrimers, optionally via linking groups, so as to
15 form polydendric assemblages (i.e., where $x>1$). Uses of these starburst bridged dendrimers include topical controlled release agents, radiation synovectomy, and others.

20 As used herein, "associated with" means that the carried material(s) can be encapsulated or entrapped within the core of the dendrimer, dispersed partially or fully throughout the dendrimer, or attached or linked to the dendrimer, or any combination
25 thereof. The association of the carried material(s) and the dendrimer(s) may optionally employ connectors and/or spacers to facilitate the preparation or use of the starburst conjugates. Suitable connecting groups
30 are groups which link a targeting director (i.e., T) to the dendrimer (i.e., P) without significantly impairing the effectiveness of the director or the effectiveness of any other carried material(s) (i.e., M) present in the starburst conjugate. These connecting groups may
35 be cleavable or non-cleavable and are typically used in order to avoid steric hindrance between the target

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director and the dendrimer, preferably the connecting groups are stable (i.e., non-cleavable). Since the size, shape and functional group density of the starburst dendrimers can be rigorously controlled, there are many ways in which the carried material can be associated with the dendrimer. For example, (a) there can be covalent, coulombic, hydrophobic, or chelation type association between the carried material(s) and entities, typically functional groups, located at or near the surface of the dendrimer; (b) there can be covalent, coulombic, hydrophobic, or chelation type association between the carried material(s) and moieties located within the interior of the dendrimer; (c) the dendrimer can be prepared to have an interior which is predominantly hollow allowing for physical entrapment of the carried materials within the interior (void volume), wherein the release of the carried material can optionally be controlled by congesting the surface of the dendrimer with diffusion controlling moieties; or (d) various combinations of the aforementioned phenomena can be employed.

Dendrimers, herein represented by "P", include the dense star polymers described in U. S. Patent 4,507,466, 4,558,120, 4,568,737 or 4,587,329.

Carried pharmaceutical materials, herein represented by "M", which are suitable for use in the starburst conjugates include any materials in vivo or in vitro use for diagnostic or therapeutic treatment which can be associated with the dense star dendrimer without appreciably disturbing the physical integrity of the dendrimer, for example, drugs such as antibiotics, analgesics, hypertensives, cardiotonics, and the like such as acetaminaphen, acyclovir, alkeran,

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amikacin, ampicillin, aspirin, bisantrene, bleomycin, neocardioastatin, chloroambucil, chloramphenicol, cytarabine, daunomycin, doxorubicin, fluorouracil, gentamycin, ibuprofen, kanamycin, meprobamate, 5 methotrexate, novantrone, nystatin, oncovin, phenobarbital, polymyxin, probucol, procarbazine, rifampin, streptomycin, spectinomycin, symmetrel, thioguanine, tobramycin, trimethoprim, valban; and toxins such as diphtheria toxin, gelonin, exotoxin A, 10 abrin, modeccin, ricin, or toxic fragments thereof; metal ions such as the alkali and alkaline-earth metals; radionuclides such as those generated from actinides or lanthanides or other similar transition elements or from other elements, such as ^{67}Cu , ^{90}Y , 15 ^{111}In , ^{131}I , ^{186}Re , ^{105}Rh , $^{99\text{m}}\text{Tc}$, ^{67}Ga , ^{153}Sm , ^{159}Gd , ^{175}Yb , ^{177}Lu , ^{88}Y , ^{166}Ho , $^{115\text{m}}\text{In}$, ^{109}Pd , ^{82}Rb , ^{194}Ir , ^{140}Ba , ^{149}Pm , ^{199}Au , ^{140}La , and ^{188}Re ; signal generators such as fluorescing entities; signal 20 reflectors such as paramagnetic entities for example ^{56}Fe , Gd , ^{55}Mn ; chelated metal such as any of the metals given, whether or not they are radioactive when associated with a chelant; signal absorbers such as electron beam opacifiers; antibodies including 25 monoclonal antibodies and anti-idiotypic antibodies; antibody fragments; hormones; biological response modifiers such as interleukins, interferons, viruses and viral fragments; diagnostic opacifiers; and 30 fluorescent moieties. Carried pharmaceutical materials include scavenging agents such as chelants, antigens, antibodies or any moieties capable of selectively scavenging therapeutic or diagnostic agents.

35 Preferably the carried pharmaceutical materials are bioactive agents. As used herein, "bioactive"

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refers to an active entity such as a molecule, atom, ion and/or other entity which is capable of detecting, identifying, inhibiting, treating, catalyzing, controlling, killing, enhancing or modifying a targeted entity such as a protein, glycoprotein, lipoprotein, lipid, a targeted cell, a targeted organ, a targeted organism [for example, a microorganism or animal (including mammals such as humans)] or other targeted moiety.

100

The starburst conjugates of formula (I) are prepared by reacting P with M, usually in a suitable solvent, at a temperature which facilitates the association of the carried material (M) with the starburst dendrimer (P).

15

Suitable solvents are solvents in which P and M are at least partially miscible and inert to the formation of the conjugate. If P and M are at least partially miscible with each other, no solvent may be required. When desired, mixtures of suitable solvents can be utilized. Examples of such suitable solvents are water, methanol, ethanol, chloroform, acetonitrile, toluene, dimethylsulfoxide and dimethylformamide.

20

25

The reaction conditions for the formation of the starburst conjugate of formula (I) depends upon the particular dendrimer (P), the carried pharmaceutical material (M), and the nature of the bond (*) formed. For example if P is the PEI (polyethyleneimine) starburst dendrimer with a methylene carboxylate surface, M is a radionuclide, e.g. yttrium, then the reaction is conducted at room temperature in water. However, if P is an ester terminated PAMAM starburst dendrimer, M is aspirin, then the reaction is conducted

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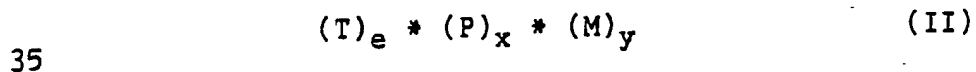
at room temperature in chloroform. Typically, the temperature can range from room temperature to reflux. The selection of the particular solvent and temperature will be apparent to one skilled in the art.

5

The ratio of M:P will depend on the size of the dendrimer and the amount of carried material. For example, the molar ratio (ratio of moles) of any ionic M to P usually is 0.1-1,000:1, preferably 1-50:1, and 10 more preferably 2-6:1. The weight ratio of any drug or toxin M to P usually is 0.1-5:1, and preferably 0.5-3:1.

When M is a radionuclide, there are three ways 15 the starburst conjugate can be prepared, namely: (1) P can be used as a chelant. For example a methylenecarboxylate surface PEI or PAMAM will chelate a metal such as yttrium or indium. (2) A chelate can be covalently bonded to P. For example, an amine 20 terminated PEI starburst dendrimer can be reacted with 1-(p-isothiocyanatobenzyl)diethylenetriaminepenta-acetic acid and then chelated, or a complex such as rhodium chloride chelated with isothiocyanatobenzyl-2,3,2-tet can be reacted. (3) A prechelated 25 radionuclide can be associated with P by hydrophobic or ionic interaction.

Particularly preferred starburst conjugates are 30 those conjugates which contain a target director (herein designated as "T") and which are represented by the formula:



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wherein

each T represents a target director;

5 e represents an integer of 1 or greater; and

P, x, *, M, and y are as previously defined herein.

Preferred among the starburst conjugates of formula
10 (II) are those in which M is a drug, radionuclide,
chelator, chelated metal, toxin, signal generator,
signal reflector, or signal absorber. Also preferred
conjugates are those conjugates in which $e=1$ or 2 ; and
those in which $x=1$ and $y=2$ or more. Particularly
15 preferred conjugates are those in which $x=1$, $e=2$, $y=2$
or more and M and T are associated with the polymer via
the same or different connectors.

* The starburst conjugates of formula (II) are
20 prepared either by forming T^*P and then adding M or by
forming P^*M and then adding T. Either reaction scheme
is conducted at temperatures which are not detrimental
to the particular conjugate component and in the
presence of a suitable solvent when required. To
25 control pH, buffers or addition of suitable acid or
base is used. The reaction conditions are dependent on
the type of association formed(*), the starburst
dendrimer used (P), the carried pharmaceutical material
30 (M), and the target director (T). For example, when T
is a monoclonal antibody and M is a radionuclide, the
 T^*P association is done through a functional group such
as an isothiocyanate in water or in water with an
organic modifier such as acetonitrile or
35 dimethylformamide. Usually, the conjugation is done in
a buffer at pH 7-10, preferably pH 8.5-9.5. The formed

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conjugate is then chelated with a radionuclide such as yttrium acetate, preferably at room temperature. Alternatively, P and M can be chelated, usually in water, before conjugation to T. The conjugation with T
5 is carried out in a suitable buffer.

The ratio of T:P is preferably 1:1, especially when T is an antibody or fragment. The ratio of M:P will be as before.

10 Target directors capable of targeting the starburst conjugates are entities which when used in the starburst conjugates of the present invention result in at least a portion of the starburst
15 conjugates being delivered to a desired target (for example, a protein, glycoprotein, lipoprotein, lipid, a targeted cell, a targeted organ, a targeted organism or other targeted moiety) and include antibodies, preferably monoclonal antibodies, antibody fragments
20 such as Fab, Fab', F(ab')₂ fragments or any other antibody fragments having the requisite target specificity, hormones, biological response modifiers; epitopes; chemical functionalities exhibiting target
25 specificity; and the like.

The antibodies or antibody fragments which may be used in preferred starburst conjugates described herein can be prepared by techniques well known in the
30 art. Highly specific monoclonal antibodies can be produced by hybridization techniques well known in the art, see, for example, Kohler and Milstein (1975, Nature 256:495-497; and 1976, Eur. J. Immunol. 6:511-519). Such antibodies normally have a highly specific
35 reactivity.

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In the antibody targeted starburst conjugates, antibodies directed against any antigen or hapten may be used. Although conventional polyclonal antibodies may be used, monoclonal antibodies offer several advantages. Each monoclonal antibody is highly specific for a single epitope. In addition, large amounts of each monoclonal antibody can be produced. Antibodies used in the present invention may be directed against, for example, tumors, bacteria, fungi, viruses, parasites, mycoplasma, differentiation and other cell membrane antigens, pathogen surface antigens, toxins, enzymes, allergens, drugs and any biologically active molecules. For a more complete list of antigens see U. S. Patent 4,193,983.

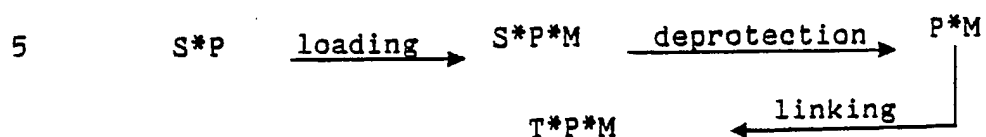
It may be desirable to connect more antibodies or fragments to the dendrimer, and in particular instances to connect antibodies of different specificities. For example, a bifunctional conjugate which has the ability to localize and bind to a tumor and then scavenge circulating cytotoxic, diagnostic, or biostatic compounds can be designed.

In the absence of a target director (or in the presence of a target director if desired), due to the number of functional groups which can be located at or near the surface of the dendrimer, all or a substantial portion of such functional groups can be made anionic, cationic, hydrophobic or hydrophilic to effectively aid delivery of the starburst conjugate to a desired target of the opposite charge or to a hydrophobic or hydrophilic compatible target.

Preparation of the conjugates of formula (II) using a P with a protected handle (S) is also intended

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as a process to prepare the conjugates of formula (II).
The reaction scheme is shown below:



10 where:

S^*P represents the protected dendrimer;

S^*P^*M represents the protected dendrimer
conjugated with M;

15 P^*M represents the dendrimer conjugated
with M (starburst conjugate);

T^*P^*M represents the starburst conjugate
linked to the target director.

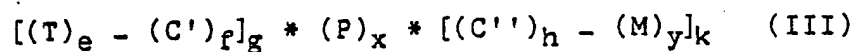
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Suitable solvents can be employed which do not
effect P^*M . For example when S is t-butoxycarbamate, S
can be removed by aqueous acid.

25

Also preferred are starburst conjugates in
which the polymer is associated directly, or via
connectors; these starburst conjugates are represented
by the formula:

30



wherein

35

each C' represents the same or different connecting
group;

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each C'' represents the same or different connecting group;

5 g, and k each individually represent an integer of 1 or greater;

f and h each individually represent an integer of 0 or greater;

10 - indicates a covalent bond in instances where a connecting group is present; and

P, x, *, M, y, T, and e are as previously defined herein.

15 Preferred among the starburst conjugates of formula (III) are those in which M is a radionuclide, drug, toxin, signal generator, signal reflector or signal absorber. Also preferred are those conjugates in which
20 x=1. Particularly preferred conjugates are those in which x, e, f, h, and y are each 1, and g is one or more and k is each individually 2 or more. Most preferred are those conjugates in which x, e, f, h, y, and g are each 1, and k is 2 or more. Also
25 particularly preferred are those starburst conjugates in which M represents a bioactive agent such as a radionuclide, drug, or toxin.

Suitable connecting groups which are
30 represented by C'' are groups which link the carried pharmaceutical material to the dendrimer without significantly impairing the effectiveness of the carried pharmaceutical material or the effectiveness of the target director(s) present in the starburst conju-
35 gate. These connectors must be stable (i.e., non-cleavable) or cleavable depending upon the mode of activity

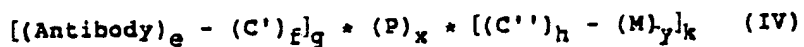
-23-

of the carried pharmaceutical material and are typically used in order to avoid steric hindrance between the carried pharmaceutical material and the polymer.

5

Most preferred are conjugates in which the dendrimer is associated directly, or via connecting group(s), to one antibody or antibody fragment. The polymer in these preferred conjugates may, in addition,
 10 be optionally associated either directly, or via connecting group(s), to one or more other carried materials, preferably a radioisotope. Such starburst conjugates are represented by the formula:

15



wherein .

20

each Antibody represents an antibody or antibody fragment capable of interacting with a desired epitope;

- indicates a covalent or coulombic bond in instances where a connecting group is present; and

25

P, x, *, M, T, e, y, C', C'', g, k, f, and h are as previously defined herein.

30

For the above synthesis of starburst dendrimers (P) which have a functional group available for linking (C' or C'') with a targeting director (T), the preferred process requires that the reactive functionality be protected as a synthetic precursor. This protection is preferred because it enables the synthesis of dendrimer or conjugates of very high quality. This process
 35 allows for the chemical binding of a unit of carried

-24-

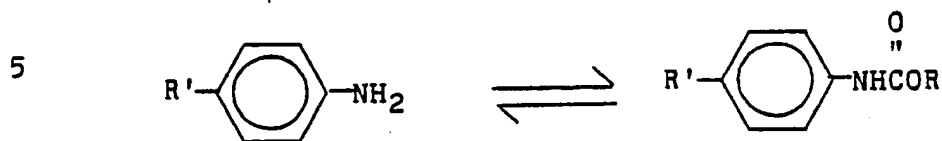
pharmaceutical material (M) to the terminal functional groups of the starburst dendrimer (P) in ways which would otherwise result also in reaction with a linking functional group, thus making it impossible to attach to the targetting director (T). Subsequent deprotection or synthetic conversion into the desired linking functional group thus enables the starburst conjugate to be linked to the targeting director.

10. One of the preferred "functional groups for linking" (hereafter referred to as a "handle") is an aniline moiety. This group is preferred because it can be used directly for linking to the targeting director, or it can be readily modified to other functional groups suitable for reaction with the targetting director, e.g. isothiocyanate, isocyanate, semithiocarbazide, semicarbazide, bromoacetamide, iodoacetamide, and maleimide. The aniline moiety is also preferred as a handle for linking with the targetting directors because it can be readily protected for use in starburst dendrimer synthesis, or the nitro group can be used as a precursor which can be converted into the desired amino function at the end of the synthesis.

There are a number of protecting groups which are suitable for protecting the anilino amino functionality during starburst dendrimer synthesis. (See Theodora W. Green, Protective Groups In Organic Synthesis, Pub. John Wiley & Son, New York, 1981). A preferred class of protecting groups are the carbamates shown below.

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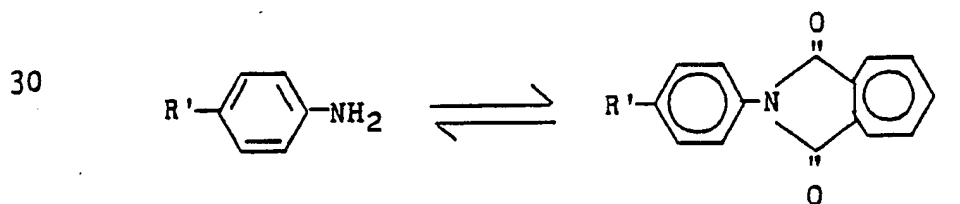


10 Many carbamates have been used for protection of amines. The most preferred carbamates for starburst dendrimer synthesis is the t-butoxycarbamate, $\text{R} = -\text{C}(\text{CH}_3)_3$. Deprotection is achieved by mild acid hydrolysis. Also preferred is the benzylcarbamate

15 protecting group, $\text{R} = -\text{CH}_2\text{-C}_6\text{H}_5$, which is preferred when the dendrimer is susceptible to acid hydrolysis. Deprotection is achieved by catalytic hydrogenation. Also preferred is the 9-fluorenylmethylcarbamate,



25 The phthalimide protecting group is also a preferred example,



35

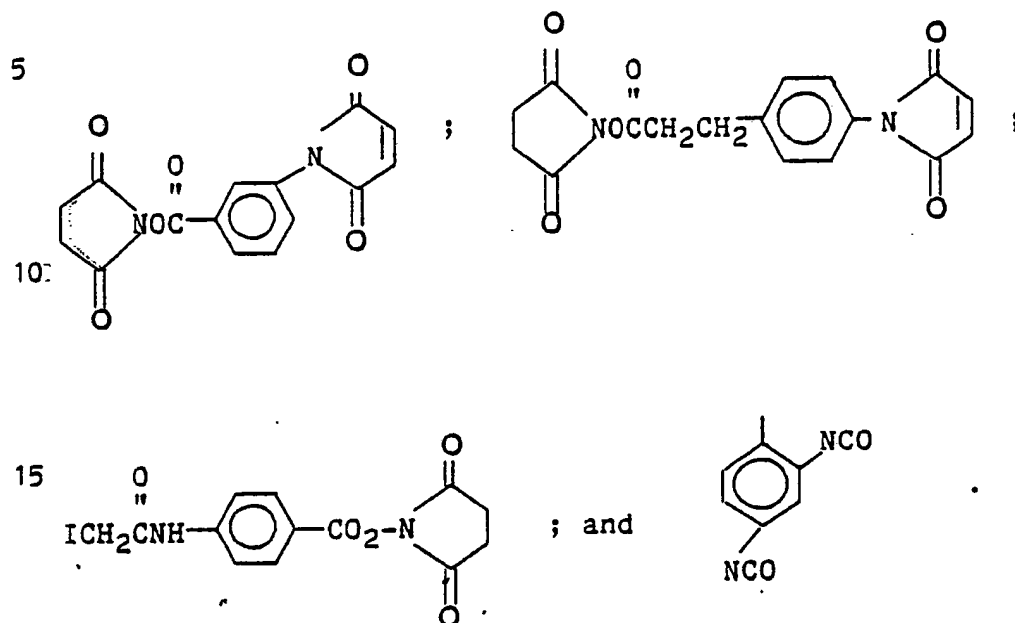
-26-

Other protecting groups used for amines which are well known in the literature could also be used in this synthetic scheme. The above preferences are given as illustrative examples only but are not the only protecting groups which can be used. Any protecting group which is stable under the reaction conditions and can be removed without altering the integrity of the starburst dendrimer can be employed.

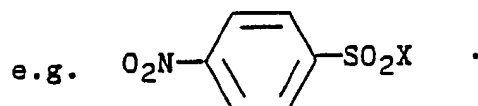
The above process can introduce an aminophenyl functionality into any agent containing an amino group which is then conjugated with some bioactive agent, e.g. monoclonal antibody or enzyme. The agent can be conjugated by oxidative coupling to carbohydrates on the bioactive agent, e.g. antibody. The aminophenyl group also can be converted into an isothiocyanate or isocyanate for subsequent reaction with the pendant amino groups of lysine residues on the bioactive agent.

An alternate process involves the reaction of an activated aryl halide, e.g. 4-nitrofluorobenzene, with an amino-function; on the agent for conjugation, e.g. starburst polyethyleneimines (PEI), and subsequent catalytic hydrogenation of the nitro group to the aniline functionality for subsequent conjugation. It is particularly useful for agents, e.g. polyamines, which need further modification prior to use, due to the relative chemical inertness of the nitrophenyl functionality to all non-reducing reaction conditions. The more common bifunctional linking agents, e.g. active esters or diisocyanates, which are reactive under a large number of reaction conditions and which would render them unusable for conjugation include:

-27-



The invention also includes the use of nitro-substituted arylsulphonyl halides to give sulphonamides,



35 The advantage of this process over known processes of introducing an aminophenyl group for conjugation is that it takes place at a late stage of the synthesis. Gansow et al., U.S. Patent 4,472,509, in his process introduced the nitrophenyl group at the

-28-

first step of a long synthetic procedure, thereby having limitations on the chemistry available.

5 This process also introduces a handle which is clearly differentiable from the remainder of the molecule. Manabe et al. disclosed that the ring opening of succinic anhydride by residual amines gave a coupling group through which conjugation to an antibody was possible. This method however gave no means of
10 differentiating between any unchelated sites on the polymer, since the chelating groups were the same as the linking group.

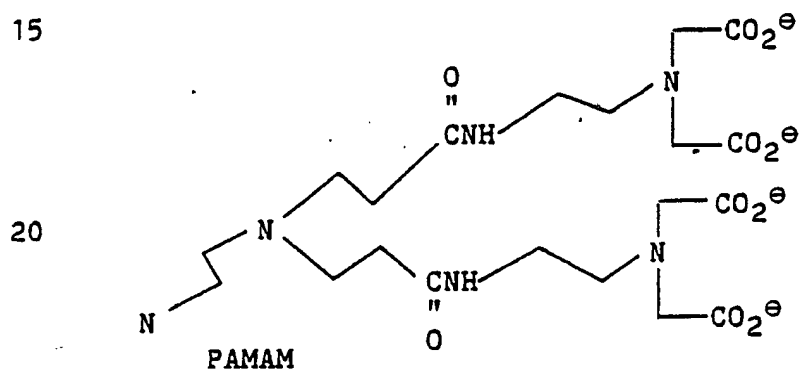
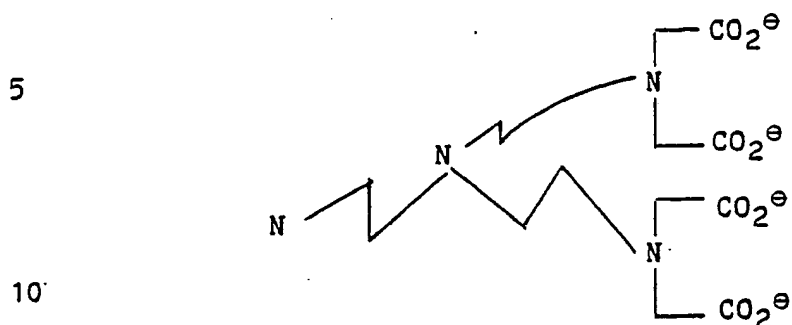
The present process also provides for direct
15 synthesis of lanthanides with starburst dendrimers, preferably by PEI acetate dendrimer. In contrast, Denkwalter, U.S. Patent 4,289,872, states that just putting acetates on the surface works. However, the present reaction shows that PEI acetate, works much
20 better than PAMAM, i.e. surface of iminodiacetates is only part of the story, the nature of the backbone, and branching is very important as well. The PEI acetate has better chelating properties than the PAMAM acetate.

25

30

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-29-



25 Preferred among the starburst conjugates of formula (IV) are those in which M is a radionuclide, drug, toxin, signal generator, signal reflector or signal absorber. Also preferred are those conjugates

30 in which $x=1$. Particularly preferred are those conjugates in which x , e , f , h , and y are each 1, and g is one or more and k is each individually 2 or more. Most preferred are those conjugates in which x , e , f , h , y , and g are each 1, and k is 2 or more. Also

35 particularly preferred are those starburst conjugates in which "Antibody" represents a monoclonal antibody or

-30-

an epitope binding fragment thereof; and especially preferred are those in which M represents a bioactive agent such as a radionuclide, drug, or toxin.

5 The starburst conjugates can be used for a variety of in vitro or in vivo diagnostic applications such as radioimmunoassays, electron microscopy, enzyme linked immunosorbent assays, nuclear magnetic resonance spectroscopy, contrast imaging, and immunoscintigraphy, 10 in analytical applications, in therapeutic applications as a carrier of antibiotics, radionuclides, drugs or other agents suitable for use in the treatment of disease states such as cancer, autoimmune diseases, 15 central nervous system disorders, infectious diseases, and cardiac disorders, or used as starting materials for making other useful agents.

 The present invention is also directed to starburst conjugate compositions in which the starburst 20 conjugates are formulated with other suitable vehicles. The starburst conjugate compositions may optionally contain other active ingredients, additives and/or diluents.

25 The preferred starburst polymer for use in the starburst conjugates of the present invention is a polymer that can be described as a starburst polymer having at least one branch (hereinafter called a core 30 branch), preferably two or more branches, emanating from a core, said branch having at least one terminal group provided that (1) the ratio of terminal groups to the core branches is more than one, preferably two or greater, (2) the density of terminal groups per unit 35 volume in the polymer is at least 1.5 times that of an extended conventional star polymer having similar core

-31-

and monomeric moieties and a comparable molecular weight and number of core branches, each of such branches of the extended conventional star polymer bearing only one terminal group, and (3) a molecular
5 volume that is no more than about 80 percent of the molecular volume of said extended conventional star polymer as determined by dimensional studies using scaled Corey-Pauling molecular models. As used herein, the term "dense" as it modifies "star polymer" or
10 "dendrimer" means that it has a smaller molecular volume than an extended conventional star polymer having the same molecular weight. The extended conventional star polymer which is used as the base for comparison with the dense star polymer is one that has
15 the same molecular weight, same core and monomeric components and same number of core branches as the dense star polymer. By "extended" it is meant that the individual branches of the conventional star polymer
20 are extended or stretched to their maximum length, e.g., as such branches exist when the star polymer is completely solvated in an ideal solvent for the star polymer. In addition while the number of terminal groups is greater for the dense star polymer molecule
25 than in the conventional star polymer molecule, the chemical structure of the terminal groups is the same.

Dendrimers used in the conjugates of the present invention can be prepared by processes known in
30 the art. The above dendrimers, the various coreactants and core compounds, and process for their preparation can be as defined in U. S. Patent 4,587,329.

The dendrimers, for use in the conjugates of
35 the present invention, can have terminal groups which are sufficiently reactive to undergo addition or

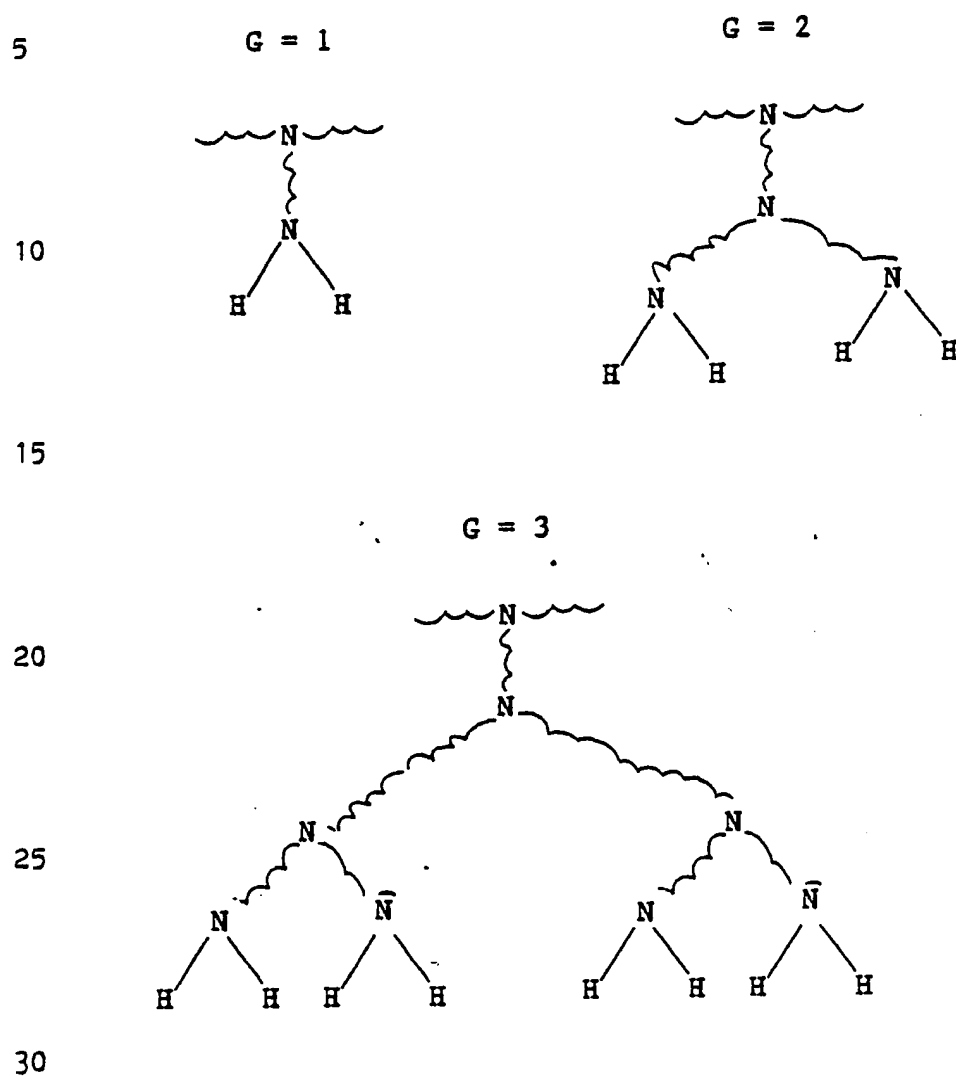
-32-

substitution reactions. Examples of such terminal groups include amino, hydroxy, mercapto, carboxy, alkenyl, allyl, vinyl, amido, halo, urea, oxiranyl, aziridinyl, oxazolinyl, imidazolinyl, sulfonato, 5 phosphonato, isocyanato and isothiocyanato. The terminal groups can be modified to make them biologically inert, for example, to make them non-immunogenic or to avoid non-specific uptake in the liver, spleen or other organ. The dendrimers differ 10 from conventional star or star-branched polymers in that the dendrimers have a greater concentration of terminal groups per unit of molecular volume than do conventional extended star polymers having an 15 equivalent number of core branches and an equivalent core branch length. Thus, the density of terminal groups per unit volume in the dendrimer usually is at least 1.5 times the density of terminal groups in the conventional extended star polymer, preferably at least 20 5 times, more preferably at least 10 times, most preferably from 15 to 50 times. The ratio of terminal groups per core branch in the dense polymer is preferably at least 2, more preferably at least 3, most preferably from 4 to 1024. Preferably, for a given 25 polymer molecular weight, the molecular volume of the dense star polymer is less than 70 volume percent, more preferably from 16 to 60, most preferably from 7 to 50 volume percent of the molecular volume of the 30 conventional extended star polymer.

Preferred dendrimers for use in the conjugates of the present invention are characterized as having a univalent or polyvalent core that is covalently bonded 35 to dendritic branches. Such ordered branching can be

-33-

illustrated by the following sequence wherein G indicates the number of generations:



Mathematically, the relationship between the number (#) of terminal groups on a dendritic branch and the number of generations of the branch can be represented as follows:

35

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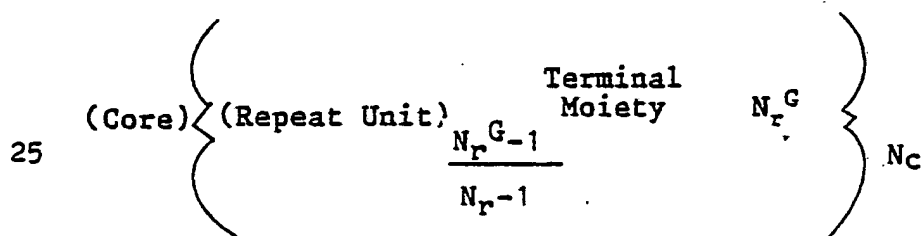
$$\# \text{ of terminal groups per dendritic branch} = \frac{N_r^G}{2}$$

5 wherein G is the number of generations and N_r is the repeating unit multiplicity which is at least 2 as in the case of amines. The total number of terminal groups in the dendrimer is determined by the following:

$$10 \quad \# \text{ of terminal groups per dendrimer} = \frac{N_c N_r^G}{2}$$

15 wherein G and N_r are as defined before and N_c represents the valency (often called core functionality) of the core compound. Accordingly, the dendrimers of this invention can be represented in its component parts as follows:

20



30 wherein the Core, Terminal Moiety, G and N_c are as defined before and the Repeat Unit has a valency or functionality of $N_r + 1$ wherein N_r is as defined before.

35 A copolymeric dendrimer which is a preferred dendrimer for the purposes of this invention is a unique compound constructed of polyfunctional monomer

-35-

units in a highly branched (dendritic) array. The dendrimer molecule is prepared from a polyfunctional initiator unit (core compound), polyfunctional repeating units and terminal units which may be the same or different from the repeating units. The core compound is represented by the formula $\textcircled{\text{I}} (\text{Z}^{\text{C}})_{\text{Nc}}$ wherein $\textcircled{\text{I}}$ represents the core, Z^{C} represents the functional groups bonded to $\textcircled{\text{I}}$ and Nc represents the core functionality which is preferably 2 or more, most preferably 3 or more. Thus, the dendrimer molecule comprises a polyfunctional core, $\textcircled{\text{I}}$ bonded to a number (Nc) of functional groups, Z^{C} , each of which is connected to the monofunctional tail of a repeating unit, $\text{X}^1\text{Y}^1(\text{Z}^1)_{\text{N}^1}$, of the first generation and each of the Z groups of the repeating unit of one generation is bonded to a monofunctional tail of a repeating unit of the next generation until the terminal generation is reached.

20 In the dendrimer molecule, the repeating units are the same within a single generation, but may differ from generation to generation. In the repeating unit, $\text{X}^1\text{Y}^1(\text{Z}^1)_{\text{N}^1}$, X^1 represents the monofunctional tail of the first generation repeating unit, Y^1 represents the moiety constituting the first generation, Z^1 represents the functional group of the polyfunctional head of the repeating unit of the first generation and may be the same as or different from the functional groups of the core compound, $\textcircled{\text{I}} (\text{Z}^{\text{C}})_{\text{Nc}}$, or other generations; and N^1 is a number of 2 or more, most preferably 2, 3 or 4, which represents the multiplicity of the polyfunctional head of the repeating unit in the first generation. Generically, the repeating unit is represented by the formula $\text{X}^i\text{Y}^i(\text{Z}^i)_{\text{N}^i}$ wherein "i" represents the

-36-

particular generation from the first to the t-1 generation. Thus, in the preferred dendrimer molecule, each Z^1 of the first generation repeating unit is connected to an X^2 of a repeating unit of the second generation and so on through the generations such that each Z^i group for a repeating unit $X^i Y^i (Z^i)_{N^i}$ in generation number "i" is connected to the tail (X^{i+1}) of the repeating unit of the generation number "i+1". The final or terminal of a preferred dendrimer molecule comprises terminal units, $X^t Y^t (Z^t)_{N^t}$ wherein t represents terminal generation and X^t , Y^t , Z^t and N^t may be the same as or different from X^i , Y^i , Z^i and N^i except that there is no succeeding generation connected to the Z^t groups and N^t may be less than two, e.g., zero or one. Therefore the preferred dendrimer has a molecular formula represented by

$$\left(\textcircled{I} (Z^c)_{N^c} \right) \left\{ \left(X^i Y^i (Z^i)_{N^i} \right)_{N^c \cap N^n}^{i-1} \right\}_{n \text{ is } 1} \left(X^t Y^t (Z^t)_{N^t} \right)_{N^c \cap N^n}^{t-1}_{n \text{ is } 1}$$

where i is 1 to t-1

wherein the symbols are as previously defined. The n function is the product of all the values between its defined limits. Thus

$$\prod_{n=1}^{i-1} N^n = (N^1)(N^2)(N^3) \dots (N^{i-2})(N^{i-1})$$

-37-

which is the number of repeat units, $X^i Y^i (Z^i)_{N^i}$,
comprising the i th generation of one dendritic branch
and when i is 1, then

5

$$\pi^0 = 1 \quad .$$
$$n=1$$

10

In copolymeric dendrimers, the repeat unit for one
generation differs from the repeat unit in at least one
other generation. The preferred dendrimers are very
symmetrical as illustrated in structural formulas
15 described hereinafter. Preferred dendrimers may be
converted to functionalized dendrimers by contact with
another reagent. For example, conversion of hydroxyl
in the terminal generation to ester by reaction with an
20 acid chloride gives an ester terminally functionalized
dendrimer. This functionalization need not be carried
out to the theoretical maximum as defined by the number
of available functional groups and, thus, a
functionalized dendrimer may not have high symmetry or
25 a precisely defined molecular formula as is the case
with the preferred dendrimer.

In a homopolymeric dendrimer, all of the repeat
units, $X^i Y^i (Z^i)_{N^i}$, are identical. Since the values of
30 all N^i are equal (defined as N_r), the product function
representing the number of repeat units reduces to a
simple exponential form. Therefore, the molecular
formula may be expressed in simpler form as:

35

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$$5 \quad \left(\textcircled{I} (Z^c)_{N_c} \right) \left\{ \left(X^i Y^i (Z^i)_{N_i} \right)_{N_c N_r^{i-1}} \right\} \left(X^t Y^t (Z^t)_{N_t} \right)_{N_c N_r^{(t-1)}}$$

10

where $i = 1$ to $t-1$

15 This form still shows the distinction between the different generations i , which each consist of $N_c N_r^{(i-1)}$ repeating units, $X^i Y^i (Z^i)_{N_i}$. Combining the generations into one term gives:

$$\left(\textcircled{I} (Z^c)_{N_c} \right) \left(X^r Y^r (Z^r)_{N_r} \right)_{N_c \frac{N_r^{(t-1)} - 1}{N_r - 1}} \left(X^t Y^t (Z^t)_{N_t} \right)_{N_c N_r^{(t-1)}}$$

or

$$\left(\textcircled{I} (Z^c)_{N_c} \right) \left\{ \left(X^r Y^r (Z^r)_{N_r} \right)_{\frac{N_r^{(t-1)} - 1}{N_r - 1}} \left(X^t Y^t (Z^t)_{N_t} \right)_{N_r^{(t-1)}} \right\}_{N_c}$$

Core

Repeat Unit

Terminal Unit

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wherein $X^r Y^r (Z^r)_{N_r}$ is the repeating unit which is used in all generations i .

Consequently, if a polymer compound will fit into these above formulae, then the polymer is a starburst polymer. Conversely, if a polymer compound will not fit into these above formulae, then the polymer is not a starburst polymer. Also, to determine whether a polymer is a starburst polymer, it is not necessary to know the process by which it was prepared, but only whether it fits the formulae. The formulae also demonstrate the generations (G) or tiering of dendrimers.

Clearly, there are several ways to determine the ratio of agent (M) to dendrimer (P) which depend upon how and where the association of P*M occurs. When there is interior encapsulation, the weight ratio of M:P usually is 10:1, preferably 8:1, more preferably 5:1, most preferably 3:1. The ratio can be as low as 0.5:1 to 0.1:1. When interior stoichiometry is used, the weight ratio of M:P is the same as for interior encapsulation. When exterior stoichiometry is determined, the mole/mole ratio of M:P is given by the following formulae:

	M	:	P
30	(A) $5 N_c N_t N_r^{G-1}$		1
	(B) $3 N_c N_t N_r^{G-1}$		1
	(C) $1 N_c N_t N_r^{G-1}$		1

where N_c means the core multiplicity, N_t means the terminal group multiplicity, and N_r means branch

-40-

junction multiplicity. The $N_c N_t N_r G^{-1}$ term will result in the number of Z groups. Thus, for example, (A) above may result when proteins, enzymes or highly charged molecules are on the surface; (B) above when it is aspirin or octanoic acid; (C) above when converting surface ester groups to carboxylate ions or groups.

Of course other structures of various dimensions can be readily prepared by one skilled in the art by appropriately varying the dendrimer components and number of generations employed. A roughly scaled comparison of three different dendrimer series relative to an IgG antibody is seen in Figure 3. The series of drawings indicated by Figure 3(B) I shows the starburst polyamidoamines (PAMAM); by II shows the starburst polyethers (PE); and by III shows the starburst polyethyleneimines (PEI). In a manner similar to that of Figure 1, all three series (I, II and III) have their far left drawing showing the initiator core, the next drawing from the left showing the starbranch oligomer, and the remaining drawings showing the starburst oligomers and respective starburst bridged dendrimers. It can be seen that in these series of scale drawings that the dendrimer dimensions are close to those noted for the IgG antibody Figure 3(A). The IgG antibody is shown to the far left in Figure 3. The scale is $1 \text{ mm} = 3.5 \text{ \AA}$. In Figure 3(A) the variable region is shown by (A); the constant region by (B); and the carbohydrate attachment sites by (C). Approximate measurements shown on Figure 3 are (1) is $35\text{--}40 \text{ \AA}$; (2) is 70 \AA ; and (3) is 60 \AA . These dimensional properties are preferred for instance where targeting involves exiting from the vascular system. Therefore dendrimer diameters of 125 Angstrom

-41-

units or less are particularly preferred in that they may allow exiting from the vascular system to targeted organs serviced by continuous or fenestrated capillaries. These dimensions are significant in that they are small compared to the size of a potential targeting component such as an antibody (see Figure 3). A linear polymer of comparable molecular weight would have a radius of gyration; (in its fully extended form), that would be much larger than the same molecular weight dendrimer. A linear polymer of this type would be expected to adversely affect the molecular recognition properties of many accepted targeting components. It is also desirable that the conjugates be of minimum molecular volume so as not to discourage, extravasation, e.g., by coupling Fab, Fab' or other appropriate antibody fragment to low molecular volume dendrimers.

Dendrimers are desirable for the delivery of radionuclides or strongly paramagnetic metal ions to tumor sites because of their ability to chelate a number of metal ions in a small volume of space. Coupling to antibodies or antibody fragments which are specific for tumors may deliver a number of metals per antibody, with only a single modification to the antibody.

Linking target directors to dendrimers is another aspect of the present invention. In preferred embodiments of the present invention, particularly where it is desired to use an antibody as a target director, a reactive functional group such as a carboxyl, sulfhydryl, reactive aldehyde, reactive olefinic derivative, isothiocyanato, isocyanato, amino, reactive aryl halide, or reactive alkyl halide can

-42-

conveniently be employed on the dendrimer. The reactive functional groups can be introduced to the dendrimer using known techniques, for example:

- 5 (1) Use of a heterofunctional initiator (as a starting material for synthesizing the dendrimer) which has incorporated into it functional groups of different reactivity. In such heterofunctional initiator at least one of the functional groups will serve as an
10 initiation site for dendrimer formation and at least one of the other functional groups will be available for linking to a target director but unable to initiate dendrimer synthesis. For example, use of protected
15 aniline to allow further modification of NH_2 groups within the molecule without reacting the aniline NH_2 .

.. The functional group which will be available for linking to a target director may be part of the initiator molecule in any one of three forms; namely:
20

- (a) In the form in which it will be used for linking with the target director. This is possible when none of the synthetic steps involved in the dendrimer synthesis can
25 result in reaction at this center.

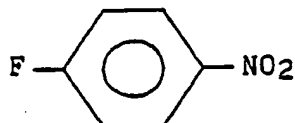
- (b) When the functional group used for linking to the targeting director is reactive in the synthetic steps involved in the
30 dendrimer synthesis, it can be protected by use of a protecting group, which renders the group unreactive to the synthetic procedures involved, but can
35 itself be readily removed in a manner

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which does not alter the integrity of the remainder of the macromolecule.

5 (c) In the event that no simple protecting group can be formed for the reactive functionality to be used for linking with the targeting director, a synthetic precursor can be used which is unreactive in all the synthetic procedures used in 10: the dendrimer synthesis. On completion of the synthesis, this functional group must be readily convertible into the desired linking group in a manner which does not alter the integrity of the remainder of 15 the molecule.

(2) Coupling (covalently) the desired reactive functional group onto a preformed dendrimer, the reagent used must contain a functionality which is 20 readily reacted with the terminal functional groups of the dendrimer. The functional group to be ultimately used to link with the targetting agent can be in its final form, as a protected functionality, or as a synthetic precursor. The form in which this linking 25: functionality is used depends on its integrity during the synthetic procedure to be utilized, and the ability of the final macromolecule to withstand any conditions necessary to make this group available for linking. 30 For example, the preferred route for PEI uses

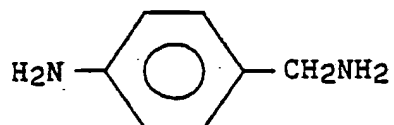


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Examples of heterofunctional initiators for use in (1) above, include the following illustrative examples:

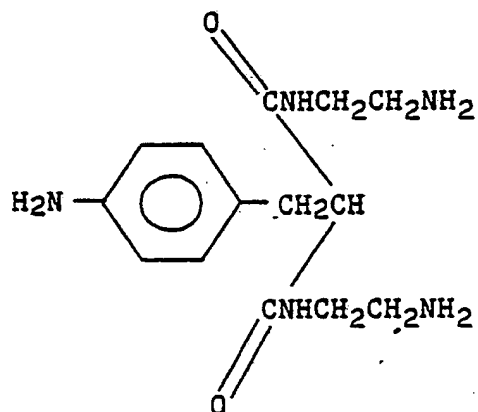
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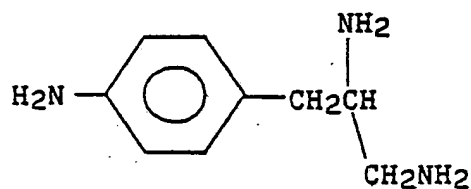
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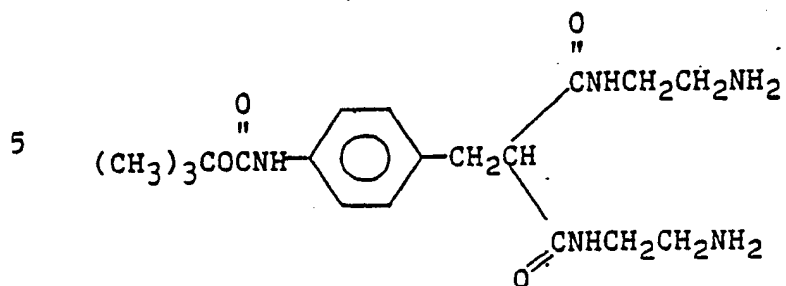


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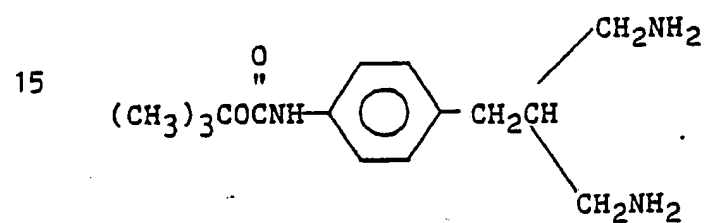
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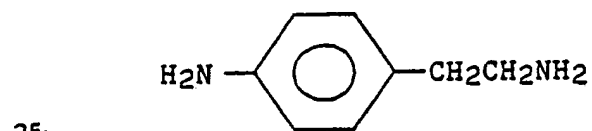
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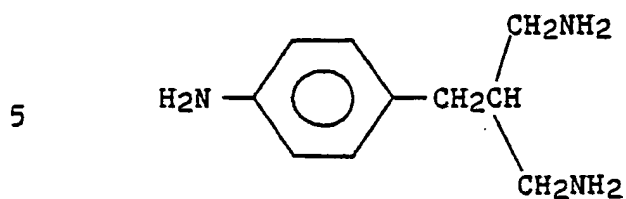


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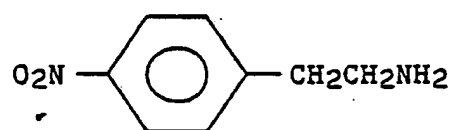
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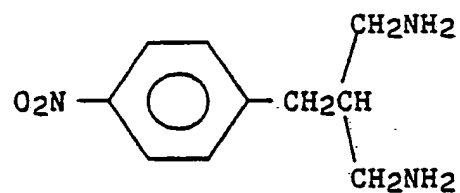


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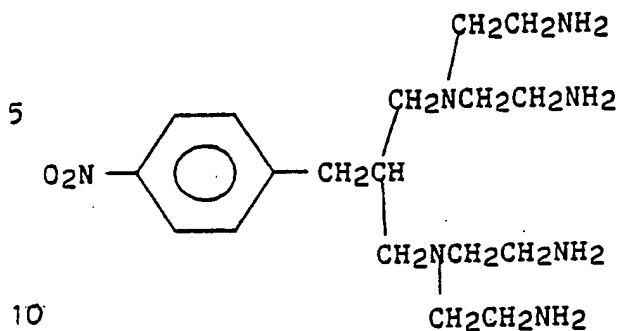
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; and

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There are several chemistries of particular importance:

- 1) Starburst Polyamidoamides ("PAMAM") Chemistry;
- 20 2) Starburst Polyethyleneimines ("PEI") Chemistry;
- 3) Starburst PEI compound with a surface of PAMAM;
- 4) Starburst polyether ("PE") chemistry.

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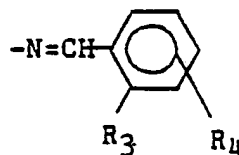
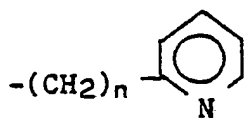
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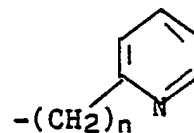
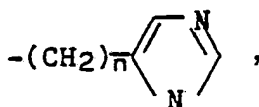
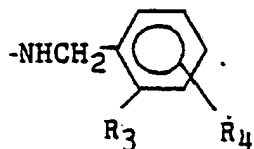
Modifications of the dendrimer surface functionalities may provide other useful functional groups such as the following:

- 5 -OPO₃H₂, -PO₃H₂, -PO₃H⁽⁻¹⁾, -PO₃⁽⁻²⁾, -CO₂⁽⁻¹⁾, -SO₂H, -SO₂⁽⁻¹⁾, -SO₃H, -SO₃⁽⁻¹⁾, -NR¹R², -R⁵, -OH, -OR¹, -NH₂, perfluorinated alkyl, -CNHR¹, -COH,
- " "
- O O

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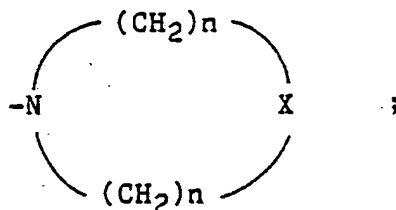
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wherein

R represents alkyl, aryl or hydrogen;

R¹ represents alkyl, aryl, hydrogen, or

25

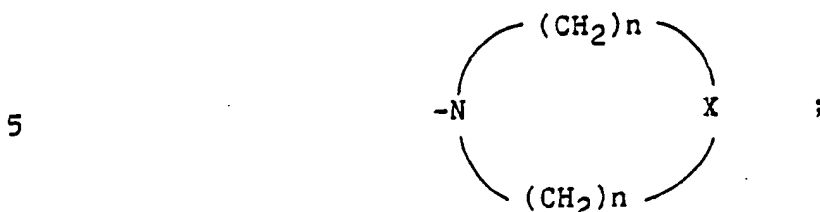


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R² represents alkyl, aryl, or



R³ represents -OH, -SH, -CO₂H, -SO₂H, or -SO₃H;

10 R⁴ represents alkyl, aryl, alkoxy, hydroxyl, mercapto, carboxyl, nitro, hydrogen, bromo, chloro, iodo, or fluoro;

R⁵ represents alkyl;

15 X represents NR, O or S; and

n represents the integer 1, 2 or 3;

polyethers; or other immuno insensitive moieties..

20 The choice of functional group depends upon the particular end use for which the dendrimer is designed.

Linking of antibodies to dendrimers is another aspect of the present invention. Typically, the antibodies or antibody fragments are linked to the dendrimer by techniques well known in the art such as attachment between a functional group on the dendrimer and moieties such as carbohydrate, amino, carboxyl, or sulfhydryl functionalities present in the antibody or antibody fragment. In some instances connecting groups may be used as connectors or spacers between the dendrimer and antibody or antibody fragment. The attachment of the dendrimer to the antibody or antibody fragment should be performed in a manner which does not significantly interfere with the immunoreactivity of

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-50-

the antibody or antibody fragment, that is, by binding the antibody or antibody fragment via a functionality in the antibody or antibody fragment which is not a part of the antigen recognition and binding site.

5

The following examples further illustrate the invention but are not to be construed as a limitation on the scope of the invention. The lettered examples concern the preparation of starting materials; the
10 numbered examples concern the preparation of products.

Example A: Preparation of 2-Carboxamido-3-(4'-nitro-phenyl)-propanamide.

15

p-Nitrobenzyl malonate diethylester (2.4 grams (g), 8.13 mmole) was dissolved in 35 ml of methanol. The solution was heated to 50-55°C with stirring and a stream of anhydrous ammonia was bubbled through the solution for 64 hours. The solution was cooled and the
20 white, flocculant product was filtered and recrystallized from 225 milliliters (ml) of boiling methanol to afford 1.85 g (7.80 mmole) of bis amide in 96% yield (mp = 235.6°C(d)).

25

The structure was confirmed by MS, ¹H and ¹³C NMR spectroscopy.

Anal: Calc. for C₁₀H₁₁O₄N₃

30

	<u>C</u>	<u>H</u>	<u>N</u>
Theo:	50.63	4.69	17.72
Found:	50.75	4.81	17.94

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Example B: Preparation of 1-Amino-2-(aminomethyl)-3-(4'-nitrophenyl)propane.

2-Carboxamido-3-(4'-nitrophenyl)propanamide (2.0 g, 8.43 mmole) was slurried in 35 ml of dry tetrahydrofuran under a nitrogen atmosphere with stirring. To this mixture was added borane/tetrahydrofuran complex (106 ml, 106 mmole) via syringe. The reaction mixture was then heated to reflux for 48 hours during which time the suspended amide dissolved. The solution was cooled and the tetrahydrofuran was removed in vacuo using a rotary evaporator. The crude product and borane residue was dissolved in 50 ml of ethanol and this solution was purged with anhydrous hydrogen chloride gas. The solution was refluxed for 1 hour and the solvent removed in vacuo. The crude hydrochloride salt was dissolved in 15 ml of deionized water and extracted with two 50 ml portions of methylene chloride. The aqueous layer was cooled in an ice bath under an argon blanket and 50% sodium hydroxide was slowly added until basic pH=11.7. The basic aqueous layer was extracted with four 25 ml portions of methylene chloride and these combined extracts were evaporated (rotary) to give 1.45 g of amber colored oil. This oil was triturated with diethyl ether (50 ml) and filtered under pressure through a short silica gel (grade 62 Aldrich) column. The column was washed with 100 ml of ether and the combined filtrates were vacuum evaporated giving 1.05 g (5.02 mmole) of the titled diamine as a clear oil (mp = 275-278°C(d) bis HCl salt).

The structure was confirmed by MS, ¹H and ¹³C NMR spectroscopy.

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Anal.: Calc. for $C_{10}H_{17}N_3O_2Cl_2$

	<u>C</u>	<u>H</u>	<u>N</u>
Theo:	42.57	6.07	14.89
Found:	43.00	6.14	15.31

5 Example C: Preparation of 1-Amino-2-(aminomethyl)-3-(4'-aminophenyl)propane.

Borane/tetrahydrofuran solution (70 ml, 70 mmole) was added under nitrogen via a cannula needle to
10 a flask containing 4-amino-benzyl malonamide (1.5 g, 7.24 mmole) with stirring. The solution was brought to reflux for 40 hours. The colorless solution was cooled and excess tetrahydrofuran was removed by rotary
15 evaporation leaving a clear gelatinous oil. Methanol (50 ml) was cautiously added to the oil with notable gas evolution. Dry hydrogen chloride was bubbled through the suspension to effect dissolution and the solution was then refluxed for 1 minute. The
20 methanol/HCl was rotary evaporated and the resulting hydrochloride salt was carried through the same dissolution/reflux procedure again. The hydrochloride salt obtained was dissolved in 10 ml of water and cooled in an ice bath under argon. Concentrated sodium
25 hydroxide (50%) was added slowly with stirring to pH=11. The aqueous portion was then extracted with 2 X 100 ml portions of chloroform which were combined and filtered through a short silica gel plug without
30 drying. The solvent was removed in vacuo (rotary) affording the title compound (0.90 g, 5.02 mmole) in 70% yield ($R_f=0.65$ - $CHCl_3/MeOH/NH_4OH$ conc - 2/2/1). The structure was confirmed by 1H and ^{13}C NMR and used without further purification.

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Example D: Preparation of 6-(4-Aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane.

4-Aminobenzyl malonate dimethylester (2.03 g, 8.43 mmole) was dissolved in 10 ml of methanol. This solution was added dropwise to a stirred solution of freshly distilled ethylene diamine (6.00 g, 103.4 mmole) in 10 ml of methanol under nitrogen over a 2 hour period. The clear solution was stirred for 4 days and Thin Layer Chromatography (TLC) analysis indicated total conversion of diester ($R_f = 0.91$) to the bis amide ($R_f = 0.42$ - 20% conc NH_4OH /80% ethanol). This material was strongly ninhydrin positive. The methanol and excess diamine were removed on a rotary evaporator and the resulting white solid was vacuum dried (10⁻¹ mm, 50°C) overnight to afford crude product (2.45g, 8.36 mmole) in 99% yield. An analytical sample was recrystallized from chloroform/hexane, MP = 160-161°C. The mass spectral, ¹H and ¹³C NMR data were consistent with the proposed structure.

Example E: Reaction of Mesityl Aziridine with 1-Amino-2-(aminomethyl)-3-(4-nitrophenyl)propane.

1-Amino-2-(aminomethyl)-3-(4-nitrophenyl)-propane (400 mg, 1.91 mmole, >96% pure) was dissolved in 10.5 ml of absolute ethanol under nitrogen. Mesityl aziridine (950 mg, 7.85 mmole) was added to the stirred diamine solution as a solid. The reaction was stirred at 25°C for 14 hours using a magnetic stirrer and during this period a white, gummy residue formed on the sides of the flask. The ethanol was decanted and the residue was triturated with another 15 ml portion of ethanol to remove any unreacted aziridine. The gummy product was

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vacuum dried (10¹mm, 25°C) to afford the tetrakis methyl sulfonamide (1.0 g, 1.44 mmole) in 75% yield (R_f = 0.74 - NH₄OH/ethanol - 20/80). The structure was confirmed by ¹H and ¹³C nuclear magnetic resonance (NMR) spectroscopy.

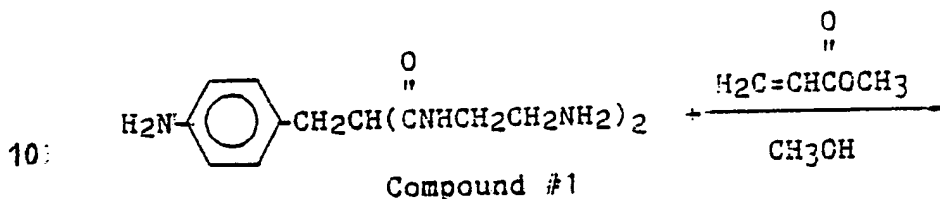
Example F: Preparation of 2-(4-Nitrobenzyl)-1,3-(bis-N,N-2-aminoethyl)diaminopropane.

The crude methylsulfonamide (650 mg, 0.94 mmole) was dissolved in 5 ml of nitrogen purged, concentrated sulfuric acid (98%). This solution was maintained under nitrogen and heated to 143-146°C for 27 minutes with vigorous stirring. A slight darkening was noted and the cooled solution was poured into a stirred solution of ether (60 ml). The precipitated white salt cake was filtered and immediately dissolved in 10 ml of deionized water. The pH of the solution was adjusted to pH=11 with 50% NaOH under argon with cooling. The resulting solution was mixed with 90 ml of ethanol and the precipitated inorganic salts were filtered. The solvent was removed from the crude amine under reduced pressure and to the resulting light brown oil was added 190 ml of toluene under nitrogen. The mixture was stirred vigorously and water was removed through azeotropic distillation (Dean-Stark trap) until the remaining toluene acquired a light yellow color (30-40 ml remaining in pot). The toluene was cooled and decanted from the dark, intractable residues and salt. This solution was stripped of solvent in vacuo and the resulting light yellow oil was vacuum dried (0.2 mm, 35°C) overnight affording 210 mg of the product (60%) which was characterized by MS, ¹H and ¹³C NMR.

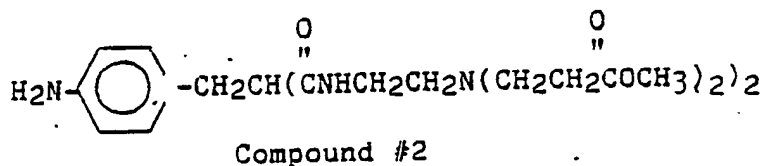
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Example G: Preparation of a starburst polymer (containing an aniline derivative) of one half generation represented by the following scheme:

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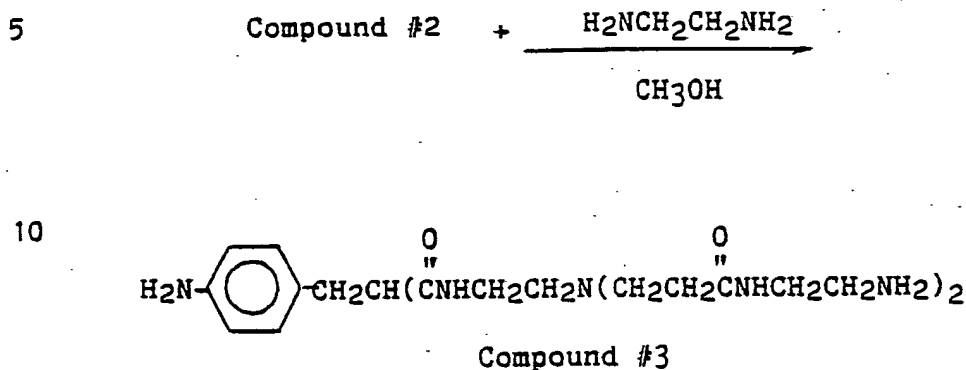
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Methyl acrylate (2.09 g, 24 mmole) was dissolved in methanol (15 ml). The compound 6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane (1.1 g, 3.8 mmole) (i.e., Compound #1) was dissolved in methanol (10 ml) and was added slowly over 2 hours with rigorous stirring to the methyl acrylate solution. The reaction mixture was stirred for 48 hours at ambient temperatures. The solvent was removed on the rotary evaporator maintaining the temperature below 40°C. The ester (Compound #2) was obtained as a yellow oil (2.6 g). No carboxyethylation of the aniline function was observed.

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Example H: Preparation of a starburst polymer (containing an aniline moiety) of one generation; represented by the following scheme:

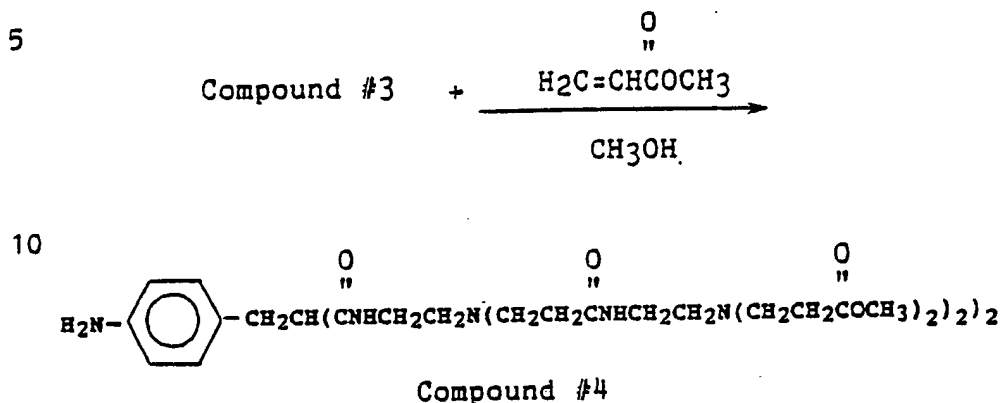


15 The ester (Compound #2) (2.6 g, 3.7 mmole) was dissolved in methanol (100 ml). this was carefully added to a vigorously stirring solution of ethylene diamine (250 g, 4.18 mole) and methanol (100 ml) at
20 such a rate that the temperature did not rise above 40°C. After complete addition the reaction mixture was stirred for 28 hours at 35-40°C (heating mantle). After 28 hours no ester groups were detectable by infrared spectroscopy. The solvent was removed on the rotary
25 evaporator at 60°C. The excess ethylene diamine was removed using a ternary azeotrope of toluene-methanol-ethylene diamine. Finally all remaining toluene was azeotroped with methanol. Removal of all the methanol
30 yielded 3.01 g of the product (Compound #3) as an orange glassy solid.

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Example I: Preparation of a starburst polymer (containing an aniline moiety) of one and one half generations represented by the following scheme:



15

The amine (Compound #3) (2.7 g, 3.6 mmole) was dissolved in methanol (7 ml) and was added slowly over one hour to a stirred solution of methyl acrylate (3.8 g, 44 mmole) in methanol (15 ml) at ambient

20 temperatures. A slight warming of the solution was observed during the addition. The solution was allowed to stir at ambient temperatures for 16 hours. The solvent was removed on the rotary evaporator at 40°C.

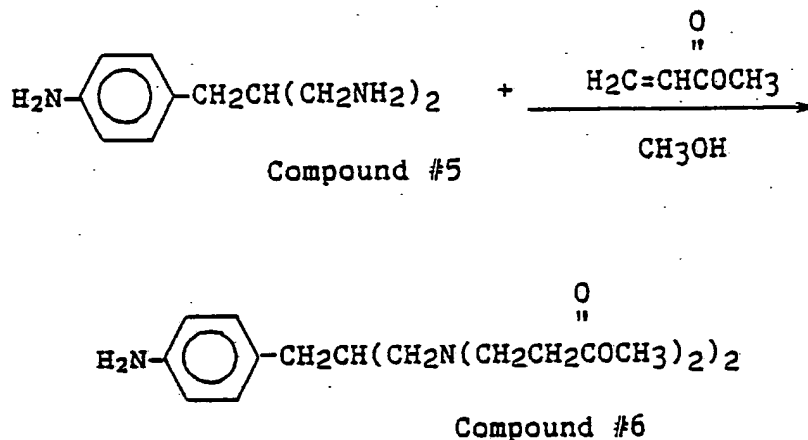
25 After removal of all the solvent and excess methyl acrylate the ester (Compound #4) was obtained in 4.7 g yield as an orange oil.

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Example J: Preparation of a starburst polymer (containing an aniline moiety) of one half generation represented by the following scheme:



20 The triamine (Compound #5, the preparation of this compound is shown in Example C) (0.42 g, 2.3 mmole) was dissolved in methanol (10 ml) and was added dropwise over one hour to methyl acrylate (1.98 g, 23 mmole) in methanol (10 ml). The mixture was allowed to stir at ambient temperatures for 48 hours. The solvent was removed on the rotary evaporator, maintaining the temperature at no higher than 40°C. The excess methyl acrylate was removed by repeated azeotropeing with methanol. The ester (Compound #6) was isolated as an orange oil (1.24 g).

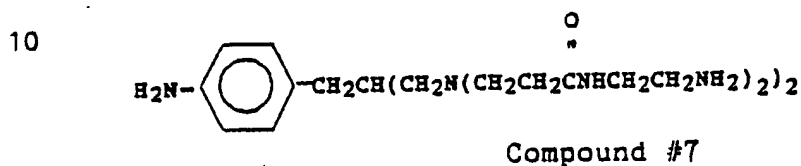
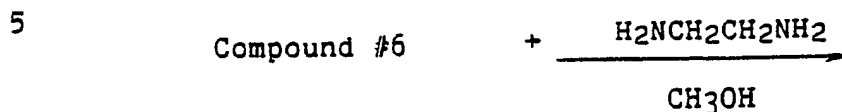
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Example K: Preparation of a starburst polymer (containing an aniline moiety) of one generation; represented by the following scheme:



15 The ester (Compound #6) (1.24 g, 2.3 mmole) was dissolved in methanol (50 ml) and was added dropwise over two hours to ethylenediamine (73.4 g, 1.22 mole) in methanol (100 ml). A small exotherm was noted,
20 vigorous stirring was maintained. The solution was left to stir at ambient temperatures for 72 hours. The solvent was removed on the rotary evaporator at 60°C. The excess ethylene diamine was removed using a ternary azeotrope of toluene-methanol-ethylenediamine. Finally
25 all remaining toluene was removed with methanol and then pumping down with a vacuum pump for 48 hours gave the amine (Compound #7) (1.86 g) as a yellow/orange oil.

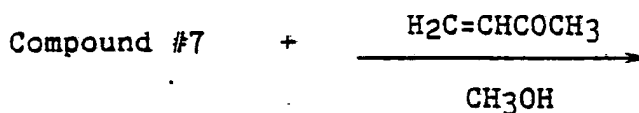
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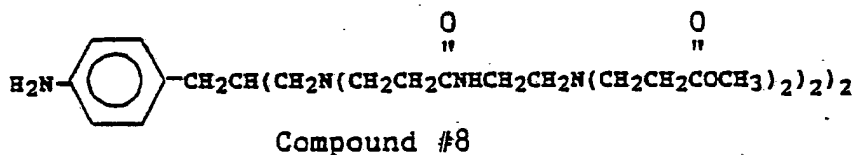
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Example L: Preparation of a starburst polymer (containing an aniline moiety) of one and one half generations; represent by the following scheme:

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The amine (Compound #7) (1.45 g, trace of methanol remained) was dissolved in methanol (100 ml) and was added slowly over 1½ hours to a stirred solution of methyl acrylate (5.80 g) in methanol (20 ml). The solution was allowed to stir for 24 hours at room temperature. Removal of the solvent followed by repeated azeotroping with methanol enabled the removal of all the excess methyl acrylate. After pumping down on a vacuum pump for 48 hours the ester (Compound #8) was isolated as an orange oil (2.50 g, 1.8 mmole).

Example M: Hydrolysis of 4.5 generation dendrimer and preparation of calcium salt.

4.5 Generation PAMAM (ester terminated, initiated off NH₃) (2.11 g, 10.92 meq) was dissolved in 25 ml of methanol and to it was added 10% NaOH (4.37 ml, 10.92 meq) (pH = 11.5-12). After 24 hours at room temperature, the pH was about 9.5. After an additional

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20 hours, the solution was rotovaped (rotary evaporated), 50 ml of toluene added, and rotovaped again.

5 The resulting oil was dissolved in 25 ml of methanol and precipitated as a white gum upon addition of 75 ml of diethyl ether. The liquid was decanted, and the gum was rotovaped to give a very fine off-white powder which upon further drying gives 2.16 g of
10 product (98% yield). No ester groups were found upon NMR and infrared analysis.

15 The sodium salt of 4.5 Generation PAMAM (ester terminated, initiated from NH_3) was replaced by the calcium salt via dialysis. The sodium salt (1.03 g) was dissolved in 100 ml of water and passed through hollow fiber dialysis tubing (cut off = 5000) at 3 ml/minute. The exterior of the tubing was bathed in 5% CaCl_2 solution. This procedure was then repeated.

The resulting solution was again dialyzed, this time against water, then repeated two additional times.

Evaporation provided 0.6 g of wet solid, which
25 was taken up in methanol (not totally soluble) and is
dried to give 0.45 g of off-white crystals.

30 C369H592O141N91Ca24 Calc. - 10.10% Ca++
M Wt. = 9526.3 Calc. = C-4432.1, H-601.8, O-2255.9,
N-1274.6, Ca-961.9)

Theo: C-46.5, H-6.32, N-13.38, Ca-10.10
Found: C-47.34, H-7.00, N-13.55, Ca-8.83

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Example N: Preparation of dendrimers with terminal carboxylate groups.

Half-generation starburst polyamidoamines were hydrolyzed to convert their terminal methyl ester groups to carboxylates. This generated spheroidal molecules with negative charges dispersed on the periphery. The dendrimers hydrolyzed ranged from 0.5 generation (three carboxylates) to 6.5 generation (192 carboxylates).

10

The products could be generated as Na^+ , K^+ , Cs^+ or Rb^+ salts.

Example O: N-t-butoxycarbonyl-4-aminobenzyl malonate dimethylester

15

4-Aminobenzyl malonate dimethylester (11.62 g, 49 mmol) was dissolved in 50 ml of t-butanol:water (60:40 with stirring. Di-t-butoxydicarbonate (19.79 g, 90 mmol) was added and the reaction mixture stirred overnight. The butanol was removed on the rotary evaporator, resulting in a yellow suspension of the product in water. Extraction into methylene chloride, drying (MgSO_4) and evaporation gave a yellow oil (21.05 g, contaminated by di-t-butoxydicarbonate). Recrystallization from 2-propanol:water (75:25) yield pale yellow crystals (11.1 g, 33 mmol, 67%). The structure was confirmed by ^{13}C NMR and purity checked by hplc analysis (spherisorb ODS-1, 0.05M H_3PO_4 pH 3: CH_3CN 55:45). The material was used without further purification.

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Example P: N-t-butoxycarbonyl-6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane

N-t-butoxycarbonyl-4-aminobenzyl malonate dimethylester (8.82 g 26 mmol), prepared in Example O, was dissolved in 50 ml of methanol. This solution was added dropwise (2 hours) to a solution of freshly distilled ethylenediamine (188 g 3.13 mole) and 20 ml of methanol, under a nitrogen atmosphere. The solution was allowed to stir for 24 hours. The ethylene diamine/methanol solution was removed on the rotary evaporator. The product was dissolved in methanol and toluene added. Solvent removal on the rotary evaporator gave the crude product as a white solid (10.70 g contaminated with ethylenediamine). The sample was divided into two samples for purification. Azeotropic removal of ethylenediamine with toluene, using a soxhlet extractor with sulphonated ion exchange beads in the thimble to trap the ethylenediamine, resulted in partial decomposition of the product, giving a brown oil. The remaining product was isolated as a white solid from the toluene on cooling (2.3 g approximately 50 percent). Analysis of a 10 percent solution in methanol by gas chromatography (Column, Tenax 60/80) showed no ethylenediamine detectable in the sample (<0.1 percent). The second fraction was dissolved in methanol to give a 10 percent solution (by weight) and purified from the ethylenediamine by reverse osmosis, using methanol as the solvent. (The membrane used was a Filmtec FT-30, in an Amicon TC1R thin channel separator, the ethylenediamine crossing the membrane.) The product was isolated as a white solid (2.7 g), in which no detectable amounts of ethylenediamine could be found by gas chromatography. The ^{13}C NMR data and HPLC analysis (Spherisorb ODS-1,

-64-

0.05M H_3PO_4 pH 3:CH₃CN 55:45) were consistent with the proposed structure. The product was used with no further purification.

- 5 Example Q: Preparation of a starburst dendrimer of one half generation from N-t-butoxycarbonyl-6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane

10 N-t-butoxycarbonyl-6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane (5.0 g 13 mmol), prepared in Example P, was dissolved in 100 ml of methanol. Methyl acrylate (6.12 g, 68 mmol) was added and the solution stirred at ambient temperatures for 72 hours. The reaction was monitored by HPLC (Spherisorb ODS1, 15 Acetonitrile: 0.04M Ammonium acetate 40:60) to optimize conversion to the desired product. The solution was concentrated to 30 percent solids, and methyl acrylate (3.0 g 32 mmol) was added. The reaction mixture was stirred at ambient temperatures until no partially 20 alkylated products were detectable by HPLC (24 hours). Removal of the solvent at 30°C by rotary evaporation, and pumping down at 1 mm Hg for 24 hours gave the product as yellow viscous oil, yield 7.81 g. The ¹³C NMR data was consistent with the proposed structure. 25 The product was used without further purification.

- 30 Example R: Preparation of a starburst dendrimer of one full generation from N-t-butoxycarbonyl-6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane

The half generation product (Example Q) (7.70 g, 10.45 mmol) was dissolved in 75 ml of methanol and added dropwise over 2 hours to a stirred solution of ethylenediamine (400 ml, 7.41 mol) and methanol (50 35 ml). The reaction mixture was stirred at ambient temperatures for 48 hours. The ethylenediamine and

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methanol were removed by rotary evaporation to give a yellow oil (11.8 g contaminated with ethylenediamine). The product was dissolved in 90 ml of methanol, and purified from the ethylenediamine by reverse osmosis (Filmtac FT-30 membrane and Amicon TC1R thin channel separator, methanol as solvent). After 48 hours, no ethylenediamine could be detected by gas chromatography (Column, Tenax 60/80). Removal of the solvent on the rotary evaporator, followed by pumping down on a vacuum line for 24 hours gave the product as a yellow glassy solid (6.72 g). Analysis by HPLC, PLRP-S column, acetonitrile:0.015M NaOH, 10-20 percent gradient in 20 min.) and ¹³C NMR analysis was consistent with the proposed structure.

Example S: Preparation of a starburst polymer of one and one half generation from N-t-butoxycarbonyl-6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane

The one generation product (Example R) (2.14 g, 25 mmol) was dissolved in 12.5 ml of methanol, and methyl acrylate (3.5 g, 39 mmol) in 5 ml of methanol was added. The solution was stirred at ambient temperatures for 48 hours, monitoring the progress of the reaction by HPLC (Spherisorb ODS-1, acetonitrile:0.04M ammonium acetate, 60:40). A second aliquot of methyl acrylate was added (3.5 g 39 mmol) and the reaction mixture stirred at ambient temperatures for a further 72 hours. Removal of the solvent on the rotary evaporator gave the product as a yellow oil (3.9 g) after pumping down overnight with a vacuum pump. The product was used with no further purification.

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Example T: Preparation of a starburst polymer of two full generations from N-t-butoxycarbonyl-6-(4-aminobenzyl)-1,4,8,11-tetraaza-5,7-dioxoundecane

The one and one half generation product (Example S) (3.9 g, 2.5 mmol) was dissolved in 50 ml of methanol, and was added dropwise over 2 hours to a stirred solution of ethylenediamine (600 g, 10 mol) and methanol (50 ml). The solution was stirred at ambient temperatures under an atmosphere of nitrogen for 96 hours. The ethylenediamine/methanol was removed on the rotary evaporator to give a yellow glassy solid (4.4 g contaminated with ethylenediamine). A 10 percent solution of the product was made in methanol, and purified from the ethylenediamine by reverse osmosis (membrane used as a Filmtac FT-30, in an Amicon TC1R thin channel separator) until no ethylenediamine could be detected by gas chromatography (Column, Tenax 60/80. Removal of the solvent gave the product as a yellow glassy solid (3.52 g). The ¹³C NMR data and HPLC analysis (PLRP-S column, acetonitrile:0.015 M NaOH, 10 to 20 percent gradient in 20 minutes, were consistent with the proposed structure.

Example U: Reaction of the two generation starburst with Bromoacetic Acid to give a methylene carboxylate terminated starburst dendrimer

The second generation product (Example T) (0.22 g, 0.13 mmol) was dissolved in 15 ml of deionized water and the temperature equilibrated at 40.5°C. Bromoacetic acid (0.48 g, 3.5 mmol) and lithium hydroxide (0.13 g, 3.3 mmol) were dissolved in 5 ml of deionized water, and added to the reaction mixture. The reaction pH was carefully maintained at 9, with the use of a pH stat (titrating with 0.1N NaOH), at 40.5°C overnight.

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Monitoring by reverse phase HPLC, (Spherisorb ODS-1 column, eluent 0.25 M H_3PO_4 pH 3 [NaOH]; acetonitrile 85:15) confirmed the synthesis of predominantly a single component.

5

Example V: Preparation of Isothiocyanato functionalized second generation methylene-carboxylate terminated starburst dendrimer

10 Five ml of a 2.8 mM solution of the second generation methylenecarboxylate terminated starburst dendrimer (Example U) was diluted with 20 ml water and the pH adjusted to 0.5 with concentrated hydrochloric acid. After one hour at room temperature the mixture
15 was analyzed by HPLC to verify the removal of the butoxycarbonyl group and then treated with 50 percent sodium hydroxide to bring the pH to 7. A pH stat (titrating with 0.1 N NaOH) was used to maintain the pH at 7 and 225 μl thiophosgene was added. After 15
20 minutes at room temperature the pH of the mixture was adjusted to 5 with 1N HCl. The mixture washed with chloroform (20 ml x 2) then concentrated on a rotary evaporator at reduced pressure. The residue recovered
25 0.91 g is a mixture of the isothiocyanate and salts.

Example W: Preparation of second generation starburst polyethyleneimine-methane sulfonamide

To a solution of 125 g N-methanesulfonyl-
30 aziridine in 50 ml ethanol was added 25.0 g tris(2-aminoethyl)amine. The solution was stirred at room temperature for 4 days. Water was added to the reaction mixture as needed to maintain the homogeneity of the solution. The solvent was removed by
35 distillation in vacuo to give the 2nd generation

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starburst PEI-methane sulfonamide as a yellow glass (161 g).

5 Example X: Cleavage of methane sulfonamides to form second generation starburst polyethyleneimine

 A solution of 5.0 g of second generation starburst PEI-methane sulfonamide, from Example W in 20 ml of 38 percent HCL was sealed in a glass ampoule. 10 The ampoule was heated at 160°C for 16 hours, then cooled in an ice bath and opened. The solvent was removed by distillation in vacuo and the residue dissolved in water. After adjusting the pH of the solution to greater than or equal to 10 with 50 percent 15 NaOH, the solvent was removed by distillation in vacuo. Toluene (150 ml) was added to the residue and the mixture heated at reflux under a Dean-Stark trap until no more water could be removed. The solution was filtered to remove salts and the filtrate concentrated 20 in vacuo to give 1.9 g second generation starburst PEI as a yellow oil.

25 Example Y Preparation of third generation starburst polyethyleneimine-methane sulfonamide

 To a solution of 10.1 g second generation starburst PEI, from Example X, in 100 ml ethanol was added 36.6 g N-methanesulfonylaziridine. The solution was stirred at room temperature for 1 week. Water was 30 added as needed to maintain the homogeneity of the solution. The solvent was removed by distillation in vacuo to give third generation starburst PEI-methane sulfonamide as a yellow glass (45.3 g).

35

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Example Z: Cleavage of methane sulfonamides to form 3rd generation starburst polyethyleneimine

5 The methane sulfonamide groups of third generation starburst PEI-methane sulfonamide (5.0 g), from Example Y, were removed by the same procedure as described for the second generation material in Example X to give 2.3 g third generation starburst PEI as a
10 yellow oil.

Example AA: Reaction of a third generation starburst polyethyleneimine with 4-fluoro-nitrobenzene

15 The third generation starburst polyethyleneimine (Example Z) (1.06 g, 1.2 mmol) was dissolved in 12 ml of absolute ethanol. (4-Fluoro)-nitrobenzene (120 μ l, 1.2 mmol) was added and the reaction mixture refluxed overnight. The solvent was
20 removed on the rotary evaporator, and the bright yellow oil dissolved in water. The aqueous solution was washed with chloroform to remove any unreacted (4-fluoro)-nitrobenzene. Removal of the water gave the product as a deep yellow oil (0.80 g). The ^{13}C NMR
25 spectrum was consistent with the proposed structure. (No attempt was made to determine the nature of the statistical distribution). The product was used without further purification.

30 Example BB: Reaction of the nitrophenyl derivative of the third generation starburst polyethyleneimine with glycolonitrile.

35 The nitrophenyl derivative of the third generation starburst polyethyleneimine (Example AA) (0.80 g) was dissolved in 20 ml of deionized water.

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Sodium hydroxide (2.80 g, 50 percent w/w) was added to the stirred solution, and the solution purged with nitrogen, venting through a sodium hydroxide scrubber. Glycolonitrile (2.85 ml of a 70 percent aqueous solution) was added at ambient temperatures. A yellow precipitate was observed to form after a few minutes. After two hours, the temperature was slowly raised to a reflux, and the solution maintained at a reflux with a nitrogen purge for 24 hours. Removal of the water gave the product as a yellow solid contaminated with glycolic acid and sodium hydroxide. The ^{13}C NMR spectrum was consistent with the proposed structure. The product was used without further purification.

15

Example CC: Hydrogenation of the nitrophenyl derivative to the aminophenyl methylenecarboxylate terminated third generation starburst polyethyleneimine.

The yellow solid from Example BB (1.70 g) was dissolved in 10 ml of deionized water, the resulting pH of the solution was approximately 11. Palladium on charcoal (200 mg of 5 percent Pd/C) was added to the reaction mixture in a glass Parr shaker bottle. The reaction mixture was placed under a pressure of 40 psi (275 kPa) of hydrogen, and shaken at ambient temperature in a Parr hydrogenation apparatus, for 6 hours. The reaction mixture was then filtered through a 0.5 m Millipore filter to remove the Pd/C and the solvent removed in vacuo and was gel filtered through a Biogel P2 resin (25 g swollen with water). Acidification with HCl resulted in an orange brown solution, which was purged with nitrogen overnight. Removal of the solvent in vacuo gave the product as the hydrochloride salt which was a pale brown solid (3.98 g, contaminated with NaCl and glycolic acid, maximum

-71-

theoretical amount of product 1.15g). The product was used with no further purificate.

5 Example DD: Preparation of 4-isothiocyanatophenyl methylenecarboxylate terminated third generation starburst polyethyleneimine

10 The product from Example CC (3.98 g) was dissolved in 15 ml of deionized water and an aliquot (2.5 ml) of this solution was diluted with 10 ml water. the pH of the solution was adjusted to 7 with sodium hydroxide. A pH stat (titrating with 1N NaOH) was used to maintain the pH and 200 μ l thiophosgene was added.
15 After 10 minutes the pH of the mixture was adjusted to 4 with hydrochloric acid. Water was removed on a rotary evaporator at reduced pressure (a small amount of n-butanol was added to prevent foaming) . The
20 residue was washed with methylene chloride and then dried. The crude product (0.95 g) a mixture of isothiocyanate (0.14 g) and salts was used without further purification.

25 Example EE: Preparation of a methylenecarboxylate-terminated second generation starburst polyamidoamine (initiated from ammonia)

30 The second generation starburst polyamidoamine (2.71 g, 2.6 mmol) and bromoacetic acid (4.39 g, 31.6 mmol) were dissolved in 30 ml of deionized water and the pH adjusted to 9.7 with 5N NaOH using a pH stat. The reaction was maintained at this pH for a half hour, and the temperature was slowly raised to 60°C and was
35 maintained at 60°C for three hours at constant pH. The pH was raised to 10.3, and the reaction mixture

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remained under control of the pH stat at ambient temperatures overnight. The reaction mixture was refluxed for a further four hours prior to work up. Removal of the solvent, and azeotroping the final traces of water with methanol gave the product as a pale yellow powder (8.7 g, contaminated with sodium bromide). The ^{13}C NMR spectrum was consistent with the proposed structure (with some contamination due to a small amount of defected material as a result of some monoalkylation).

Example FF: Preparation of a methylenecarboxylate terminated second generation starburst polyethyleneimine (initiated from ammonia)

The second generation starburst polyethyleneimine (2.73 g, 6.7 mmol), from Example X, and bromoacetic acid (11.29 g 81 mmol) were dissolved in 30 ml of deionized water. The pH was slowly raised to pH 9.5 maintaining the temperature below 30°C. The temperature was raised slowly to 55°C, and the reaction pH maintained at 9.5 for 6 hours with the aid of a pH stat (titrating with 5N NaOH). The pH was raised to 10.2, and maintained at that pH overnight. Removal of the solvent on the rotary evaporator, and azeotroping the final traces of water using methanol, gave the product as a yellow powder (17.9 g, contaminated with sodium bromide). The ^{13}C NMR spectrum was consistent with the proposed structure (with some contamination due to a small amount of defected material as a result of some monoalkylation).

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Example GG: Preparation of a 3.5, 4.5, 5.5 and 6.5 generation starburst PAMAM

To a 10 wt% methanolic solution of 2.46 g 3 generation PAMAM starburst was added 2.32 g of methyl acrylate. This mixture was allowed to sit at room temperature for 64 hr. After solvent and excess methyl acrylate removal, 4.82 g of product was recovered (105% of theoretical).

Preparation of higher 1/2 generation starburst PAMAM'S:

Generations 4.5, 5.5 and 6.5 were prepared as described above with no significant differences in reactant concentrations, reactant mole ratios or reaction times.

Example HH: Preparation of a 4, 5 and 6 generation starburst PAMAM:

To 2000 g of predistilled ethylenediamine was added 5.4 g of 4 1/2 generation starburst PAMAM as a 15 wt% solution in methanol. This was allowed to sit at room temperature for 48 hrs. The methanol and most of the excess ethylenediamine were removed by rotary evaporation under water aspirator vacuum at temperature less than 60°C. The total wt of product recovered was 8.07 g. Gas chromatography indicated that the product still contained 34 wt% ethylenediamine at this point. A 5.94 g portion of this product was dissolved in 100 ml methanol and ultrafiltered to remove the residual ethylenediamine. The filtration was run using an Amicon TC1R thin channel recirculating separator equipped with an Amicon YM2 membrane. An in-line pressure relief valve was used to maintain 55 psig (380 kPa) pressure

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across the membrane. The 100 ml was first concentrated to 15 ml by forcing solvent flow exclusively through the membrane. After this initial concentration, the flow was converted to a constant volume retentate recycle mode for 18 hrs. After this time, 60 ml of methanol was passed over the membrane to recover product still in the module and associated tubing. The product was stripped of solvent and 2.53 g of 5 generation starburst PAMAM was recovered. Analysis by gas chromatography indicated 0.3% residual ethylenediamine remained in the product.

Preparation of generation 4 and 6 proceeded as above with the only difference being the weight ratio of ethylenediamine to starting material. To prepare 4th generation this ratio was 200:1 and for 6th generation this ratio was 730:1.

Example 1: Incorporation of 2-(acetyloxy)benzoic acid (aspirin) into starburst dendrimers.

A widely accepted method for ascertaining whether a "probe molecule" is included in the interior of a micelle is to compare its carbon-13-spin lattice relaxation times (T_1) in a non-micellized versus micellized medium. A substantial decrease in T_1 for the micellized medium is indicative of "probe molecule" inclusion in the micelle. Since starburst dendrimers are "covalently fixed" analogs of micelles, this T_1 relaxation time technique was used to ascertain the degree/extent to which various pharmaceutical type molecules were associated with starburst polyamidoamines. In the following examples, T_1 values for (acetyloxy)benzoic acid (I) (aspirin) were

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determined in solvent (CDCl₃) and then compared to T₁ values in CDCl₃ at various [I:dendrimer] molar ratios.

5 Inclusion of aspirin (I) into various starburst polyamidoamine dendrimers as a function of generation.

Various half generation (ester terminated, initiated from NH₃) starburst polyamidoamine dendrimers (G = 0.5 → 5.5) were combined with 2-(acetyloxy)benzoic acid in CDCl₃ to give acid:tertiary amine ratios of = 1.0. A plot of T₁ values for 2-(acetyloxy)benzoic acid versus generation of starburst dendrimer added (see Figure 4 where • represent C-4, n represent C-6, and o represent C-5) shows that T₁ reaches a minimum over the generation range of 2.5 → 5.5 for carbons 4, 5 and 6 in 2-(acetyloxy)benzoic acid. This demonstrates association of 2-(acetyloxy)benzoic acid in the dendrimers (G = 2.5 → 5.5) and further confirms that polyamidoamine dendrimers (Gen = 2.5 or greater) can function as carrier molecules.

Example 2 Release of pseudoephedrine from starburst dendrimer - PAMAM

25 Pseudoephedrine (0.83 mg/ml) and starburst PAMAM dendrimer [1.0 mg/ml; G= 6.5; terminal group (Z) = 192 (methyl ester)] were dissolved in deionized distilled water and the pH of the donor phase was adjusted to 9.5, with sodium hydroxide solution, and stored at room temperature for about 12 hours. Solution of pseudoephedrine alone was treated in the same way (control). The drug dendrimer solution was stored at 40°C for 8 hours after the first experiment and dynamic dialysis performed. Dialysis membrane used was a spectra/Por 7, MWCO 1,000 28.6 mm in diameter in

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spectrum separation cells (half cell volume 5 and 10 ml, cell dimensions: 38 mm diameter for both the cells and the cell depth of 10 and 20 mm for 5 and 10 ml cells, respectively).

5

Samples were analyzed by a HPLC procedure developed for pseudoephedrine conditions for which are as follows:

10

Column: uBondapak C-18

Mobile phase: pH 3.2 phosphate buffer plus acetonitrile (80:20)

Flow rate : 0.3 ml/min

15

Detection: UV at 210 nm

Retention time: 13.3 min

The dialysis membrane was washed with deionized water and was kept soaking in the receptor phase for at least 12 hours prior to use. The dialysis membrane was placed in between the donor and the receptor compartment was stirred with a small magnetic spin bar. Known volumes of donor and receptor solutions were introduced into the respective compartments and transfer of pseudoephedrine to the receptor compartment was followed as a function of time. To maintain sink conditions the entire receptor phase was removed periodically (every 30 minutes) and replaced with fresh receptor phase. The amount of pseudoephedrine was assayed in the sampled receptor phase. Experiments were conducted at room temperature (22°C). The receptor phase was plain deionized distilled water.

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The results of dynamic analysis are shown in Figure 5. In Figure 5, the • represents pseudoephedrine only (control), the represents pseudoephedrine plus the dendrimer, and the O represents pseudoephedrine plus the dendrimer at 40°C, 8 hours before dialysis. It is apparent that in presence of G = 6.5 dendrimer in the donor compartment the rate of dialysis of pseudoephedrine is reduced. Storing the donor solution at 40°C, appears to further reduce the rate of dialysis.

10

The experiment was repeated at lower concentrations (the ratio of number of drug molecules to the number of terminal groups was kept the same).
G = 6.5 dendrimer 120 µ/ml pseudoephedrine 100 µ/ml (122 µ/ml salt).

15

Dynamic dialysis of pseudoephedrine (alone) at this lower concentration was almost identical to that at higher concentration. Figure 6 summarizes the results of this experiment where • represents pseudoephedrine only (control), and o represents pseudoephedrine plus dendrimer.

20

25 Example 3

The procedure of Example 2 was repeated using the modifications given below.

30

Receptor phase: pH 7.4 phosphate buffer

Donor phase: pH 7.4 phosphate buffer plus drug and dendrimer in the following ratios:

35

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	1.	G 6.5 : Drug	::	1 : 192
5	2.	G 5.5 : Drug	::	1 : 96
	3.	G 4.5 : Drug	::	1 : 48
	4.	G 6.5H: Drug	::	1 : 192
	5.	G 5.5H: Drug	::	1 : 96
10	6.	G 4.5H: Drug	::	1 : 48

The above donor phase compositions plus pseudoephedrine alone were subjected to dynamic dialysis. The letter "H" after the dendrimer generation number stands for hydrolyzed dendrimer.. Hydrolysis was accomplished by the procedure described in Examples M and N.

The results of these experiments are summarized in Figure 7 where the donor and receptor compartment contained pH 7.4 phosphate buffer. For pseudoephedrine alone (P) the mean curve of three experiments is plotted (shown by the solid line), and one typical run from the other experiments are shown. In Figure 7, the following symbols represent the dendrimer of the indicated generation.

30

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Table III

	Symbol	Dendrimer Generation
5	O	5.5
	●	6.5
	O	4.5
10	⊖	5.5H
	O	6.5H
	O	4.5H

15 Pseudoephedrine appears not to associate with the
dendrimer (unhydrolyzed) at pH 7.4. Hydrolysis of the
terminal functional groups into carboxylate form, has a
dramatic effect on the dialysis rate (reduction). The
20 generation number appears not to influence the dialysis
rate.

Example 4: Interaction studies of salicylic acid with
PAMAM starburst dendrimers

25 This example evaluated interaction
characteristics of salicylic acid with PAMAM starburst
dendrimers. These dendrimers consisted of an ammonia
initiated core with repeating units derived from N-(2-
30 aminoethyl) acrylamide. Both full (amine terminated
functions) and half (ester terminal groups) generation
polymers were included in the studies. The ratio of
salicylic acid to starburst dendrimers utilized in the
experiments resulted in approximately one salicylic
35 acid molecule to one terminal amine functional group
for full generation polymers. In the half-generation

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polymer study, the same ratio was employed with adjustments made for the higher molecular weight polymer.

5 The experiments were conducted at room temperature using an equilibrium static cell dialysis methodology. Spectrum static dialysis cells (half cell volume, 10 ml) separated by SpectraPor 6 membranes (molecular weight cutoff = 1000) were utilized for all
10 experiments. Transport of salicylic acid was monitored as a function of time by removing aliquots from appropriate cell compartments and assayed by HPLC analysis using a U.V. detector at 296 nm (Bondupak C-18 Column, eluting mobile phase of acetonitrile/0.1M
15 phosphate buffer (pH 3.2) at a ratio of 20:80 (V/V), set at a flow rate of 30 ml/hour).

 Ten ml of a solution containing 1 mg/ml salicylic acid and 2.5 mg/ml starburst polymer (Gen
20 4.0) adjusted to pH 6.65 and 5.0 with HCL solution were placed in the donor compartment of the dialysis cell and an equal volume of purified water adjusted to the same pH's placed in the receptor compartment.
25 Transport of salicylic acid into the receptor compartment was monitored. The results are given in Figure 8. In Figure 8, the free acid is represented by ●, the acid plus generation 4.0 dendrimer, pH 6.65 is represented by ○, and the acid plus generation 4.0
30 dendrimer, pH 5.00 is represented by □.

 Due to the lower percent ionization of the amine groups on the polymer at pH 6, a greater extent of interaction with salicylic may be expected at pH 5,
35 resulting in less compound transported at the lower pH. The results given in Figure 8 indicate a much lower

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percentage of salicylic acid transported in the presence of polymer at both pH's studied compared to the salicylic acid control study. It is also observed that more salicylic acid is transported at pH 6.65 than at pH 5.0 as predicted. The data demonstrates an interaction of the starburst polymer with salicylic acid that can be controlled by pH. Sustained release characteristics are also implied by the data since the salicylic acid levels in the presence of polymer continue to rise past the approximate 12-hour equilibrium point observed in the control study.

To further investigate the interaction characteristics of salicylic acid with starburst polymers (Gen = 4.0) an experiment was designed at pH 8.0. The design of the study differed from that previously described in that only the salicylic acid solution (1 mg/ml), adjusted to pH 8.0, was placed in the donor compartment and the polymer solution (2.5 mg/ml) placed in the receptor compartment. Loss of salicylic acid from the donor compartment was monitored as previously described. The results of the experiment are given in Figure 9. In Fig 9, the free acid is represented by \bullet , and the acid plus generation 4.0 dendrimer at pH 8.0 is represented by Δ .

As indicated in Figure 9, the equilibrium characteristics of salicylic acid in the donor compartment with starburst polymers in the receptor compartment differs from the salicylic acid control study. Based on the ionization characteristics of the molecules at pH 8, approximately 6-7% interaction is expected. The observed extent of interaction is indicated to be on the order of 4-5%. The lower

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association observed may be due to experimental variability or to an ionization constant of less than one.

5 This experiment indicates an uptake or removal of free salicylic acid from the continuous phase of the system by the polymer. This type of action could result in suppression of reactivity of molecules suggesting a possible chelating type of property
10 associated with the polymers.

 The interaction characteristics of salicylic acid at pH 6.65 with a half generation starburst polymer (Gen = 4.5) having ester terminated functional
15 groups were evaluated. Salicylic acid (1 mg/ml) was combined with starburst polymer (Gen = 4.5) 3.6 mg/ml at pH 6.65. Ten ml of the solution was placed in the donor compartment and transport from the donor compartment was monitored as previously described. The
20 results are given in Figure 10. In Figure 10, the free acid is represented by -●-, and the acid plus polymer is represented by ---o---.

 Under these experimental conditions no charge
25 interaction is predicted to occur since the tertiary amine groups are non-ionized at pH 6.65. As is indicated in Figure 10, the loss of salicylic acid in the presence of polymer (Gen = 4.5) is virtually
30 identical during the first 10 hours of dialysis to that of the salicylic acid control study.

 The following observations are made from the data presented in this example:

35

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- (1) Full generation PAMAM starburst polymers function as a carrier for salicylic acid.
- (2) Full generation PAMAM starburst polymers have sustained release functionality for salicylic acid.
- (3) Salicylic acid carrier properties of full generation PAMAM starburst polymers can be controlled by pH.

Example 5: Demonstration of multiple chelation of iron by a sodium propionate terminated sixth generation starburst polyamidoamine.

The sodium propionate terminated sixth generation polyamidoamine (initiated from ammonia) (97.1 mg, 2.45 mol.) was dissolved in 1.5 ml of deionized water. Addition of 0.5 ml of 0.5N HCl reduced the pH to 6.3. Ferric chloride was added (0.5 ml of 0.1.2M solution, 0.051 mmol) producing a light brown gelatinous precipitate. On heating at 60°C for 0.5 hours, the gelatinous precipitate became soluble, resulting in a homogeneous orange solution. The solution was filtered through Biogel P2 acrylamide gel (10 g, twice) isolating the orange band (free of halide contamination). Removal of the solvent in vacuo gave the product as an orange film (30 mg). Analysis was consistent with chelation of approximately 20 moles of ferric ions per mole of starburst dendrimer.

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Table IV

Found	Theoretical		
	Na ₄ Fe ₂₀ H ₁₂₈ SB	Na ₅ Fe ₂₀ H ₁₂₇ SB	Na ₆ Fe ₂₀ H ₁₂₆ SB
Na 0.39, 0.24 (0.31 0.1%)	0.25	0.31	0.38
Fe 3.14, 3.11 (3.12 0.02%)	3.05	3.05	3.04
C 47.11	49.87	49.84	49.81
H 7.33	7.31	7.30	7.29
N 14.81	14.49	14.48	14.47
O ----	25.03	25.02	25.01
Mwt.	36632.23	36654.21	36375.18

SB = C₁₅₂₁H₂₄₆₇N₃₇₉O₅₇₃

20 These results confirm chelation of 20 ± 2 moles of ferric ions per mole of starburst dendrimer.

Example 6: Preparation of a product containing more than one rhodium atom per starburst polymer.

25 2.5 Gen PAMAM (ester terminated, initiated from NH₃) (0.18 g, 0.087 mmole) and RhCl₃·3H₂O (0.09 g, 0.3 mmole) were mixed in dimethylformamide (DMF) (15 ml) and heated for 4 hours at 70°C. The solution turned crimson and most of the rhodium was taken up. The

30 unreacted rhodium was removed by filtration and the solvent removed on the rotary evaporator. The oil formed was chloroform soluble. This was washed with water and dried (MgSO₄) before removal of solvent to

35 yield a red oil (0.18 g). The NMR spectrum was recorded in CDCl₃ only minor differences were noted between the chelated and unchelated starburst.

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Dilution of some of this CDCl_3 solution with ethanol followed by NaBH_4 addition resulted in rhodium precipitation. $\text{RhCl}_3 \cdot 3\text{H}_2\text{O}$ is insoluble in chloroform and in chloroform starburst solution, thus confirming
5 chelation.

Example 7: Preparation of a product containing Pd chelated to a starburst polymer

3.5 Gen PAMAM (ester terminated, initiated from
10 NH_3) (1.1 g, 0.24 mmole) was dissolved with stirring into acetonitrile (50 ml). Palladium chloride (0.24 g, 1.4 mmole) was added and the solution was heated at 70-75°C (water bath) overnight. The PdCl_2 was taken up
15 into the starburst. After removal of the solvent, the NMR in CDCl_3 confirmed that chelation had occurred. Dilution of the CDCl_3 solution with ethanol and addition of NaBH_4 resulted in precipitation of the palladium. The chelated product (1.23 g) was isolated
20 as a brown oil.

Example 8: Demonstration of multiple chelation of yttrium by a methylene carboxylate terminated second generation starburst polyethyleneimine by trans
25 chelation from yttrium acetate

The starburst polyethyleneimine methylene carboxylate terminated material (0.46 g 52.5 percent active, remainder sodium bromide, 0.18 mmol active
30 starburst dendrimer), from Example FF, was dissolved in 4.5 ml of deuterium oxide. The resultant pH was 11.5-12. A solution of yttrium acetate was prepared by dissolving yttrium chloride (0.15 g, 0.5 mmol) and sodium acetate (0.41 g, 0.5 mmol) in 1.5 ml of
35 deuterium oxide (2.9 moles of yttrium per mole of dendrimer). Aliquots of 0.5 ml of the yttrium acetate

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solution were added to the dendrimer solution and the ^{13}C NMR spectra recorded at 75.5 MHz.

5 The ^{13}C NMR spectrum of yttrium acetate shows
two resonances, 184.7 ppm for the carboxyl carbon and
23.7 ppm for the methyl carbon, compared with 182.1 and
24.1 ppm for sodium acetate, and 177.7 and 20.7 ppm for
acetic acid (Sadtler ^{13}C NMR Standard Spectra).
10 Monitoring the positions of these bands indicates
degree of chelation with the starburst dendrimer. The
most informative signal for the starburst dendrimer
which is indicative of chelation is the $\alpha\text{-CH}_2$ (of the
methylene carboxylate group involved in chelation),
15 which appears at 58.4 ppm in the unchelated dendrimer,
and 63.8 ppm in the chelated dendrimer. Upon chelation
with yttrium, the spin lattice relaxation times of the
time $\alpha\text{-CH}_2$ shortens as expected from $0.24 \pm 0.01\text{s}$ to
0.14 \pm 0.01s, indicative of chelation.

20 Following the addition of 0.5 ml of the yttrium
acetate solution to the starburst dendrimer, all the
yttrium appeared to be chelated by the dendrimer,
25 confirmed by the signals for the acetate being that of
sodium acetate. The same observation was noted for the
addition of a second 0.5 ml aliquot of the yttrium
acetate solution. Upon addition of the third aliquot
of yttrium acetate, not all of the yttrium was observed
30 to be taken up as the starburst chelate, the acetate
carboxyl resonance was observed to shift to 183.8 ppm
indicating that some of the yttrium was associated with
the acetate. The integrated area of the chelated -CH_2
35 groups on the dendrimer increased, indicating that some
of the third mole equivalent of yttrium added was

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indeed chelated with the dendrimer. These results indicate that the dendrimer can chelate from 2-3 yttrium ions per dendrimer molecule.

- 5 Example 9: Demonstration of Multiple Chelation of Yttrium by a methylene carboxylate terminated second generation starburst polyamidoamine by trans chelation from yttrium acetate.

10 The same experimental methods were used for this study as were used for Example 8. The starburst polyamidoamine methylene-carboxylate terminated material (0.40g 62.5% active, remainder sodium bromide, 0.12 mmol.) was dissolved in 4-5 ml of deuterium oxide. The resultant pH was 11.5-12, which was lowered to 9.4
15 with 6N HCl prior to the experiment. A solution of yttrium acetate was prepared by dissolving yttrium chloride (0.1125g, .37 mmol.) and sodium acetate (0.0915g, 1.1 mmol.) in 1.5 ml of deuterium oxide, thus
20 every 0.5 ml of solution contains one mole equivalent of metal.

25 The first two mole equivalents of yttrium acetate added were fully chelated by the starburst polyamidoamine. On addition of a third mole equivalent of yttrium, precipitation of the product occurred and as such no NMR data could be obtained. The signals which gave the most information about chelation by the starburst dendrimer were those of the two carbons
30 adjacent to the chelating nitrogen. The chemical shifts of these carbons in the unchelated dendrimer occurred at 59.1 ppm for the α -CH₂, and 53.7 ppm for the first methylene carbon of the backbone. Upon
35 chelation these two resonances were observed to shift downfield to 60.8 and 55.1 ppm respectively. The trans chelation shows that two metal ions can be readily

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chelated per dendrimer molecule, however upon chelation of some unknown fraction of a third mole equivalent, the product precipitates out of solution.

- 5 Example 10: Demonstration of Multiple Chelation of ^{90}Y
by a methylenecarboxylate terminated second generation
starburst polyethyleneimine.

Standard solution of yttrium chloride (3×10^{-2}
M, spiked with non-carrier added ^{90}Y) and
10 methylenecarboxylate terminated second generation
starburst polyethyleneimine (6×10^{-2} M) were prepared.
These were reacted together at various metal:starburst
ratios in HEPES buffer. The complex yield was
15 determined by ion exchange chromatography using
Sephadex G50 ion exchange beads, eluting with 10%
NaCl: NH_4OH , 4:1 at pH 10. Noncomplexed metal is
removed on the column, complexed metal elutes. Yields
were obtained by comparing the radioactivity eluted
20 with that on the column, using a well counter.

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Table V

Chelation of 2.5 Gen. PEI Acetate with ^{90}Y

	<u>Vol. Y+3</u>	<u>Vol. PEI</u>	<u>Vol HEPES</u>	<u>M:L Theor.</u>	<u>% Complex</u>	<u>M:L Act.</u>
5	5	30	370	0.1	110	0.1
	10	30	360	0.2	101	0.2
	20	30	350	0.4	95	0.4
	30	35	340	0.5	97	0.5
10	30	30	340	0.5	102	0.5
	60	30	310	1.0	99	1.0
	120	30	250	2.0	100	2.0
	180	30	180	3.0	94	2.8
15	250	30	120	4.1	80	3.3
	300	20	80	7.5	44	3.3
	300	20	70	5.0	40	2.0
	300	20	70	5.0	41	2.0

20 All volumes in Table V are in microlitres

Within the accuracy of the experiments, these results indicate that the 2.5 Gen. starburst PEI acetate can chelate between 2 and 3 metals per polymer giving a

25 soluble complex.

Example 11: Conjugation of 4-isothiocyanatophenyl methylenecarboxylate terminated third generation starburst polyethyleneimine with IgG monoclonal antibody

30

The isothiocyanate, 10 mg (50 μ moles), from Example DD was dissolved in 500 μ l of 3 mM indium chloride which had been spiked with radioactive indium-111 chloride and the pH was adjusted to 9 with 660 μ l N

35 NaOH. Aliquots of whole antibody IgG CC-46 was then mixed with aliquots of the chelated starburst. The

-90-

mixtures were shaken then left for 18 hours. The mixtures were then analyzed by HPLC (column Dupont Zorbax Biosphere GF-250; eluent 0.25 M sodium acetate, pH 6) and a UV detector at 254 nm and a radioactivity detector. Results are shown in Table VI.

Table VI
Starburst-IgG Conjugates

	<u>1</u>	<u>2</u>	<u>3</u>	<u>4</u>
IgG solution (μl)	20	20	20	20
Chelated Starburst solution (μl)	5	20	50	100
% Radioactivity on IgG	6	5	5	3
% IgG conjugated	2	7	17	22

Example 12: Conjugation of 4-isothiocyanatophenyl methylenecarboxylate terminated third generation starburst polyethyleneimine with IgG monoclonal antibody

The isothiocyanate from Example DD, 4 mg (20 μ moles) was mixed with 200 μl of 3 mM indium chloride (60 μ moles). A 20 μl aliquot of the solution was then spiked with radioactive indium-111 chloride and the pH adjusted to 9 by the addition of 30 μl 1 N NaOH and 10 μl of 0.1 HCL. The indium chelate was mixed with 150 μl of CC-49 whole antibody IgG, 10 mg/ml in 50 mM HEPES buffer at pH 9.5. After 18 hours at room temperature the antibody was recovered by preparative HPLC (column Dupont Zorbax Biosphere GF 250; eluent 0.25 m sodium acetate, pH 6); and a UV detector at 254 nm and a radioactivity detector. The recovered antibody was concentrated on an Amicon membrane and exchanged into

-91-

PBS buffer at pH 7.4. The recovered antibody had specific activity of approximately 0.5 μ ci/100 μ g.

5 Example 13: In Vivo localization of ^{111}In labeled starburst antibody conjugate.

 The usefulness of the labeled starburst antibody conjugate prepared in Example 12 was
10 demonstrated by measuring the uptake of the material by a human tumor xenograft in an athymic mouse. Female athymic mice were inoculation subcutaneously with the human colon carcinoma cell line, LS-174T (approximately
15 4×10^6 cells/animal). Approximately two weeks after inoculation, each animal was injected via the tail vein. The mice were sacrificed after 17 and 48 hours (five animals at each time point), the tumor and selected tissues were excised and weighed, and
20 radioactivity was measured in a gamma counter. After 17 hours 13.5 percent of the injected dose per gram of tissue had localized at the tumor. After 48 hours 21.6% of the injected dose per gram of tissue had localized at the tumor.

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1. A starburst conjugate which comprises at least one starburst polymer associated with at least one unit of at least one carried pharmaceutical material.

2. The conjugate of Claim 1 wherein the starburst polymer is a starburst dendrimer.

5

3. The conjugate of Claim 1 or 2 wherein at least one of the carried pharmaceutical materials is a drug, radionuclide, chelant, chelated metal, toxin, antibody, antibody fragment, antigen, signal generator, signal reflector, or signal absorber.

10

4. The conjugate of Claim 2 wherein there are at least two different carried materials at least one of which is a target director and at least one of which is a bioactive agent.

15

5. The conjugate of Claim 4 wherein the target director is an entity specific for one or more target receptors and the bioactive agent is a radionuclide, drug, or toxin.

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6. The conjugate of Claim 4 or 5 wherein the target director is a polyclonal antibody or fragment thereof.

5 7. The conjugate of Claim 4 or 5 wherein the target director is a monoclonal antibody or fragment thereof.

10 8. The conjugate of Claim 1 wherein the dendrimer contains discontinuities.

9. A starburst conjugate of Claim 1 of the formula:

15 $(P)_x * (M)_y$ (I)

wherein each P represents a dendrimer;

x represents an integer of 1 or greater;

20 each M represents a unit of a carried pharmaceutical material, said carried pharmaceutical material can be the same carried pharmaceutical material or a different carried pharmaceutical material;

25 y represents an integer of 1 or greater; and

* indicates that the carried pharmaceutical material is associated with the dendrimer.

30 10. The conjugate of Claim 9 wherein M is a drug, pesticide, radionuclide, chelator, chelated metal, toxin, antibody, antibody fragment, antigen, signal generator, signal reflector, or signal absorber.

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11. The conjugate of Claim 9 wherein $x=1$ and $y=2$ or more.

5 12. The starburst conjugate of Claim 9 wherein the molar ratio of any ionic M to P is 0.1-1,000:1.

10 13. The starburst conjugate of Claim 9 wherein the weight ratio of any drug or toxin M to P is 0.1-5:1.

14. A process for preparing

15



20 wherein each P represents a dendrimer; x represents an integer of 1 or greater; each M represents a unit of a carried pharmaceutical material, said carried pharmaceutical material can be the same carried pharmaceutical material or a different carried pharmaceutical material; y represents an integer of 1
25 or greater; and * indicates that the carried pharmaceutical material is associated with the dendrimer, which comprises reacting P with M, usually in a suitable solvent, at a temperature which facilitates the association of the carried material (M)
30 with the starburst dendrimer (P).

15. The process of Claim 14 wherein the temperature is from room temperature to reflux.

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16. The process of Claim 14 wherein the
suitable solvent is water, methanol, ethanol,
chloroform, acetonitrile, toluene, dimethylsulfoxide or
dimethylformamide.

5

17. A starburst conjugate of any one of Claims
1 to 11 which also has at least one pharmaceutically
acceptable diluent or carrier present.

10

18. The starburst conjugate composition of
Claim 17 which also has other active ingredients
present.

15

19. A starburst conjugate of any one of Claims
1 to 11, 17 and 18 for use as a diagnostic agent.

20. A starburst conjugate of any one of Claims
1 to 11, 17 and 18 for use as a pharmaceutical carrier.

20

21. A method for the delivery of at least one
carried pharmaceutical material which comprises
administering of at least one starburst conjugate of
any one of Claims 1 to 11, 17 and 18 containing said
material at or near a targeted locus.

25

22. A method for scavenging therapeutic or
diagnostic compounds which comprises administering of a
bifunctional starburst conjugate containing a target
director of Claim 4 to 7 which localizes the conjugate
to a target locus and a scavenging moiety which can

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bind a secondarily administered therapeutic or diagnostic compound.

5 23. The method of Claim 22 wherein the scavenging moiety is a chelant, antigen, or antibody.

 24. A process for preparing a starburst conjugate of the formula

10



wherein

15 each C' represents the same or different connecting group;

 each C'' represents the same or different connecting group;

20 g, and k each individually represent an integer of 1 or greater;

 f and h each individually represent an integer of 0 or greater;

25 - indicates a covalent bond in instances where a connecting group is present;

30 each P represents a dendrimer;

 x represents an integer of 1 or greater;

 T represents a target director;

35 each M represents a unit (for example, a molecule, atom, ion, and/or other basic unit) of a carried

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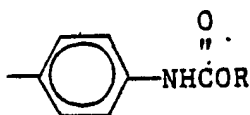
pharmaceutical material, said carried pharmaceutical material can be the same carried pharmaceutical material or a different carried material, preferably the carried pharmaceutical material is a bioactive agent;

y represents an integer of 1 or greater; and

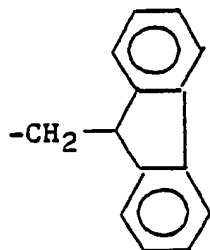
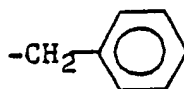
* indicates that the carried pharmaceutical material is associated with the dendrimer;

which comprises the reaction of P, having reactive moieties, with a connecting group such as an aniline moiety, which may have the NH_2 group protected.

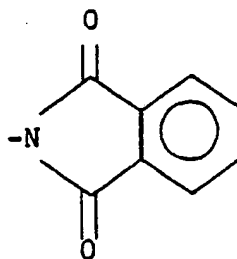
24. The process of Claim 23 wherein the protecting group has the formula



wherein R is $-\text{C}(\text{CH}_3)_3$, $-\text{CH}_2-$



or



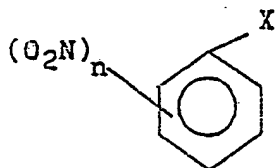
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-98-

25. The process of Claim 23 wherein the P also has a connecting group (handle) attached of the formula

5



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where n is 1 or 2, and X is F, Cl, Br, I, SO_2Cl , and when n is 1, the NO_2 group is in the para position.

26. The process of Claim 25 wherein the
15 connecting group is 4-fluoronitrobenzene.

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AMENDED CLAIMS

[received by the International Bureau on 8 January 1988 (08.01.88)
original claims 1 - 26 replaced by new claims 1 - 36 (8 pages)]

1. A starburst conjugate which comprises at least one starburst polymer associated with at least one unit of at least one carried pharmaceutical material.

2. The conjugate of Claim 1 wherein the starburst polymer is a starburst dendrimer.

5

3. The conjugate of Claim 1 or 2 wherein at least one of the carried pharmaceutical materials is a drug, radionuclide, chelator, chelated metal, toxin, antibody, antibody fragment, antigen, signal generator, signal reflector, or signal absorber.

10

4. The conjugate of Claim 2 wherein there are at least two different carried materials at least one of which is a target director and at least one of which is a bioactive agent.

15

5. The conjugate of Claim 4 wherein the target director is an entity specific for one or more target receptors and the bioactive agent is a radionuclide, drug, or toxin.

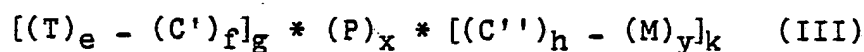
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bind a secondarily administered therapeutic or diagnostic compound.

23. The method of Claim 22 wherein the
5 scavenging moiety is a chelant, antigen, or antibody.

24. A process for preparing a starburst
conjugate of the formula
10



wherein

15 each C' represents the same or different connecting group;

each C'' represents the same or different connecting group;
20

g, and k each individually represent an integer of 1 or greater;

f and h each individually represent an integer of 0 or
25 greater;

- indicates a covalent bond in instances where a connecting group is present;

30 each P represents a dendrimer;

x represents an integer of 1 or greater;

T represents a target director;

35 each M represents a unit (for example, a molecule, atom, ion, and/or other basic unit) of a carried

pharmaceutical material, said carried pharmaceutical material can be the same carried pharmaceutical material or a different carried material, preferably the carried pharmaceutical material is a bioactive agent;

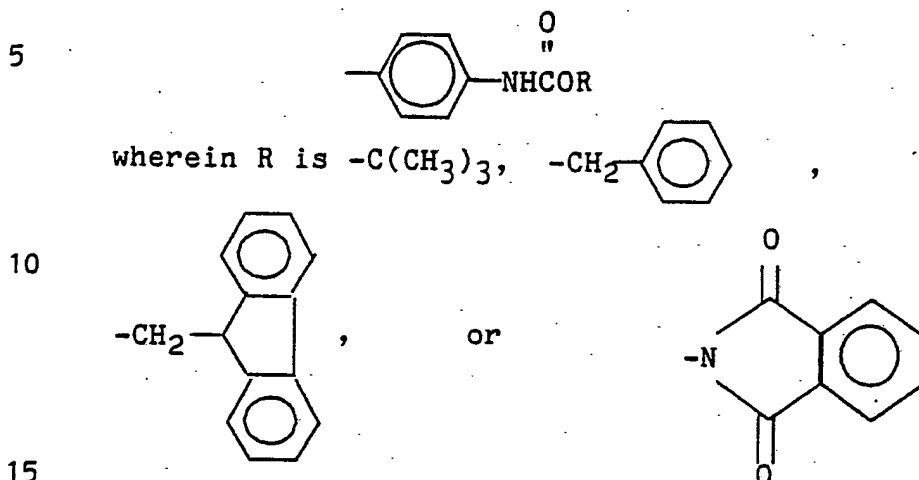
y represents an integer of 1 or greater; and

* indicates that the carried pharmaceutical material is associated with the dendrimer;

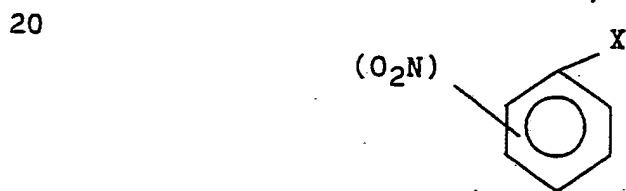
which comprises the reaction of P, having reactive moieties, with a connecting group such as an aniline moiety, which may have the NH_2 group protected.

25. A process for preparing a starburst conjugate as defined in Claim 1 which comprises the reaction of P, having reactive moieties, with a connecting group such as an aniline moiety, which may have the NH_2 group protected.

26. The process of Claim 25 wherein the protecting group has the formula



27. The process of Claim 25 wherein the P also has a connecting group (handle) attached of the formula

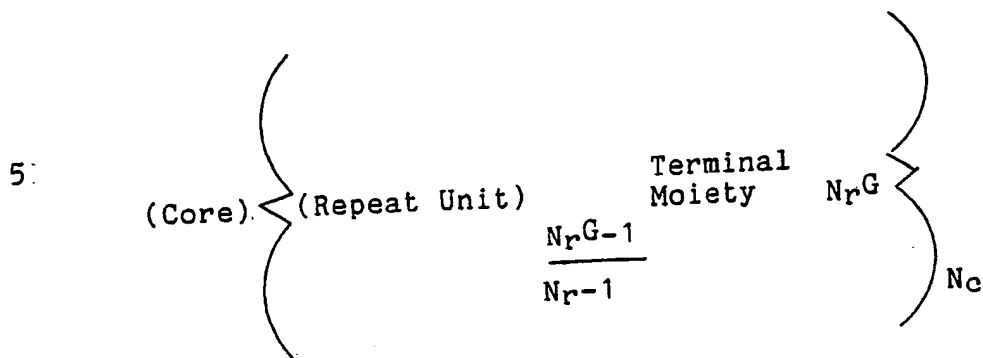


where n is 1 or 2, and X is F, Cl, Br, I, SO_2Cl , and when n is 1, the NO_2 group is in the para position.

30 28. The process of Claim 27 wherein the connecting group is 4-fluoronitrobenzene.

29. The conjugate of Claim 2 wherein the starburst dendrimer is of the formula

35



10

wherein: the core is

of terminal groups per dendritic branch =

15

$$\frac{N_r G}{2} ;$$

16 G is the number of generations; N_r is the repeating
 17 unit multiplicity which is at least 2; N_c is the
 20 valency of the core compound; the terminal moiety is
 determined by the following:

of terminal moieties per dendrimer =

25

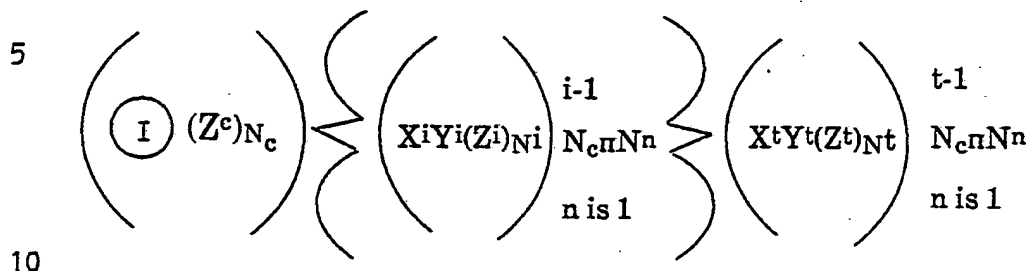
$$\frac{N_c N_r G}{2}$$

30

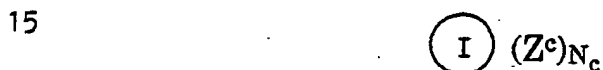
wherein N_r , G and N_c are as defined above; and the
 Repeat Unit has a valency or functionality of $N_r + 1$
 wherein N_r is as defined above.

35

30. The conjugate of Claim 2 wherein the starburst dendrimer is of the formula



wherein i is 1 to $t-1$; the core compound is represented by the formula



where



represents the core, Z^c represents the functional groups bonded to



and N_c represents the core valency; the repeat unit is represented by the formula $X^i Y^i (Z^i)_{N_i}$ wherein " i " is defined as above; the final or terminal units are represented by $X^t Y^t (Z^t)_{N_t}$ wherein t represents terminal generation and X^t , Y^t , Z^t and N_t may be the same as or different from X^i , Y^i , Z^i and N_i except that there is no succeeding generation connected to the Z^t groups and N_t may be less than two; the n function is the product of all the values between its defined limits, such as

35

i-1

$$\prod_{n=1}^{i-1} N_n = (N^1)(N^2)(N^3)\dots(N^{i-2})(N^{i-1})$$

5

n=1

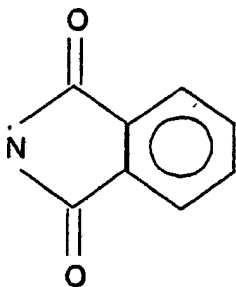
which is the number of repeat units, $X^i Y^i (Z^i) N^i$,
comprising the i th generation of one dendritic branch
and when i is 1, then $n^0 = 1$.

10

n=1

31. A process for preparing a starburst
conjugate as defined in Claim 1 which comprises the
reaction of P, having reactive moieties, with an
aniline moiety, which may have the NH_2 group protected
by an N-phthalimide of the formula

20



25

32. A process for preparing a starburst
conjugate as defined in Claim 1 which comprises the
reaction of P, having reactive moieties, which may have
the NH_2 group protected by any protecting group used
for amines which is inert under the conditions used for
starburst synthesis.

30

33. A process for preparing a starburst
polyethyleneimine which comprises reacting a starburst
polyethyleneiminemethane sulfonamide with hydrochloric
acid.

35

34. A process for purifying a starburst dendrimer having a solvent present which comprises removing the solvent by ultrafiltration using a membrane.

5

35. The process of Claim 34 wherein the solvent is ethylenediamine.

10

36. A process for preparing a starburst conjugate of Claim 1 wherein the starburst polymer is a starburst dendrimer and the carried material is a lanthanide which comprises chelation of the lanthanide with a starburst polyethyleneimine acetate.

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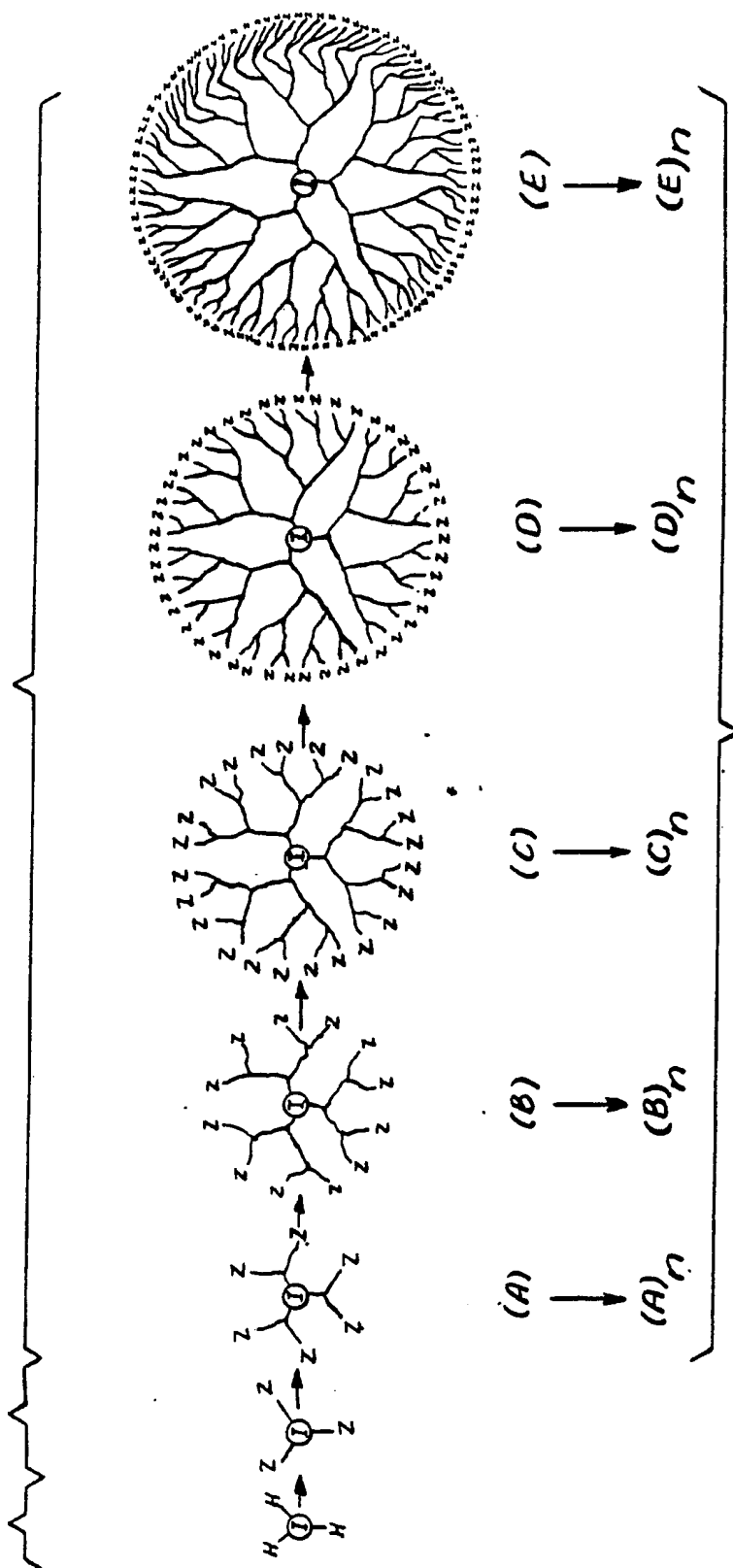


Fig. 1

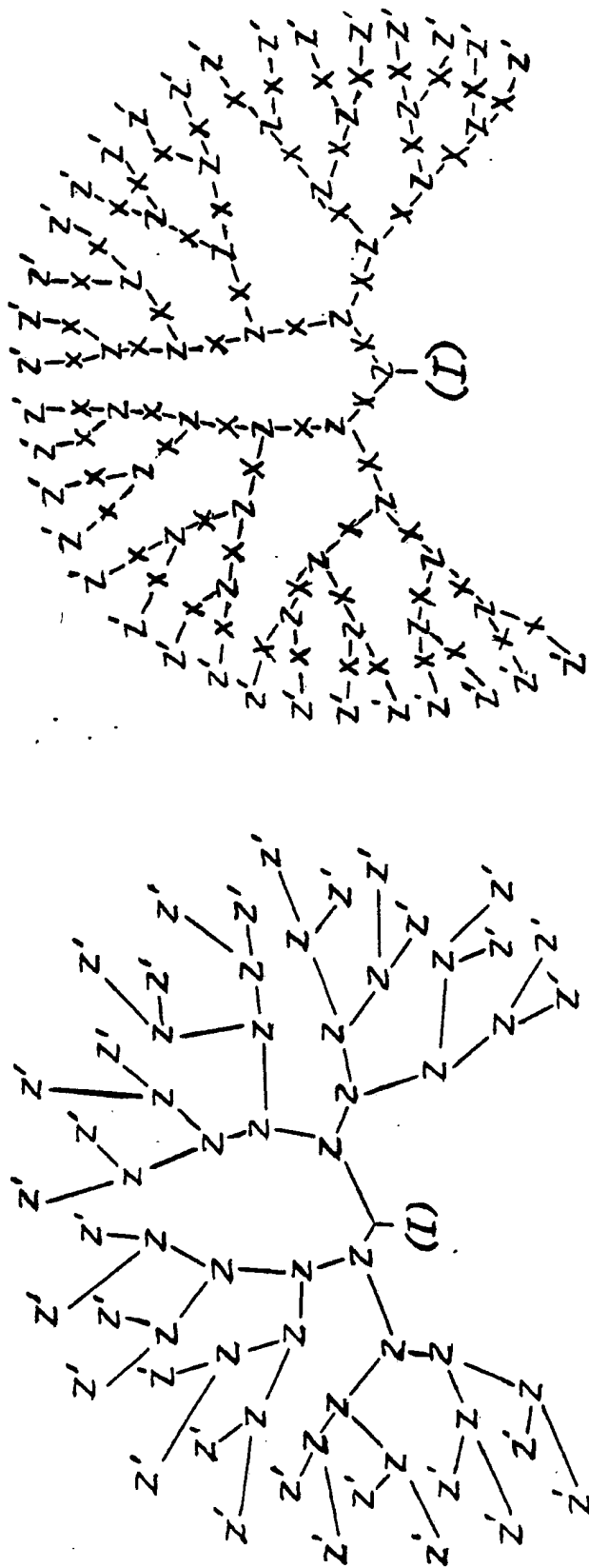


Fig. 2

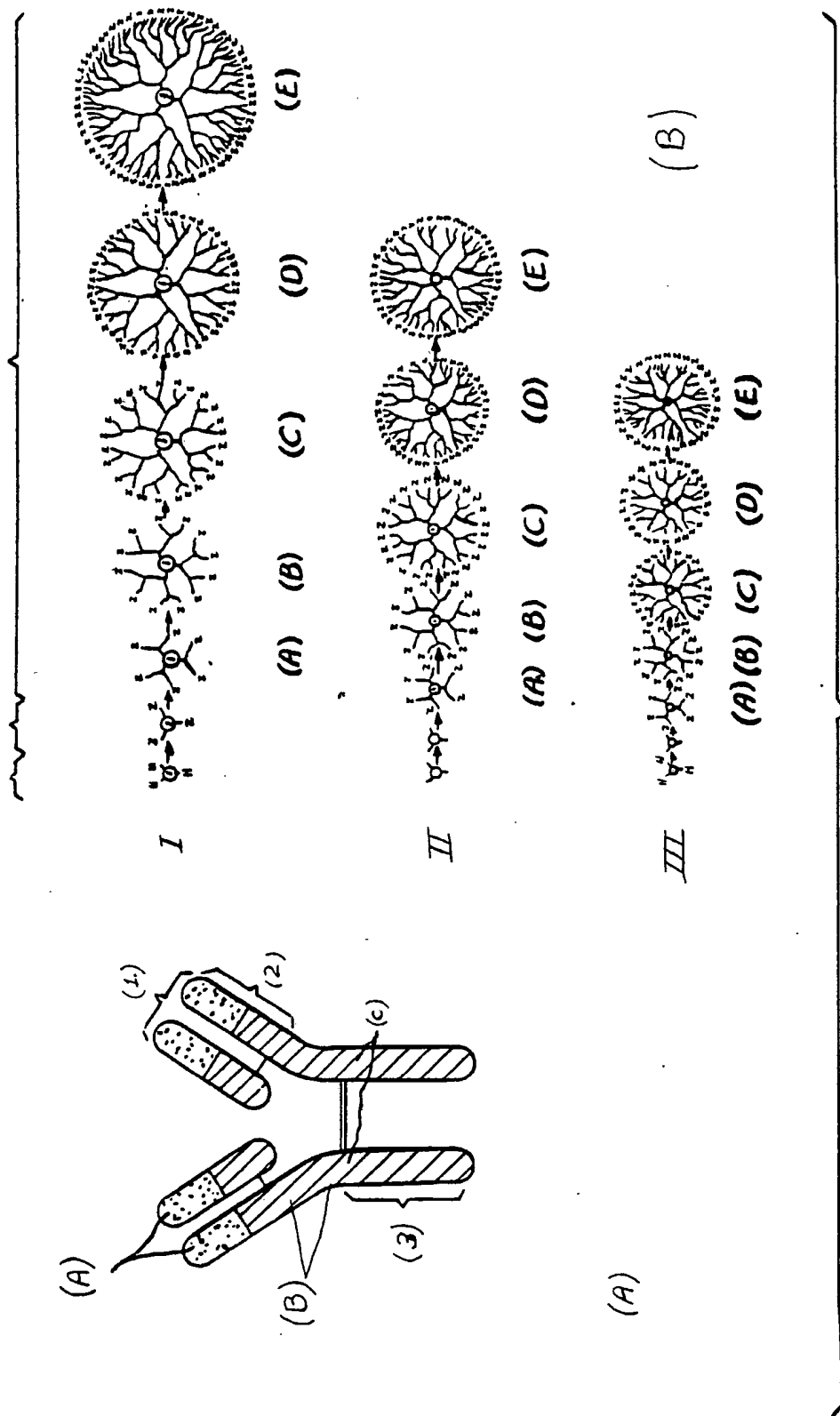
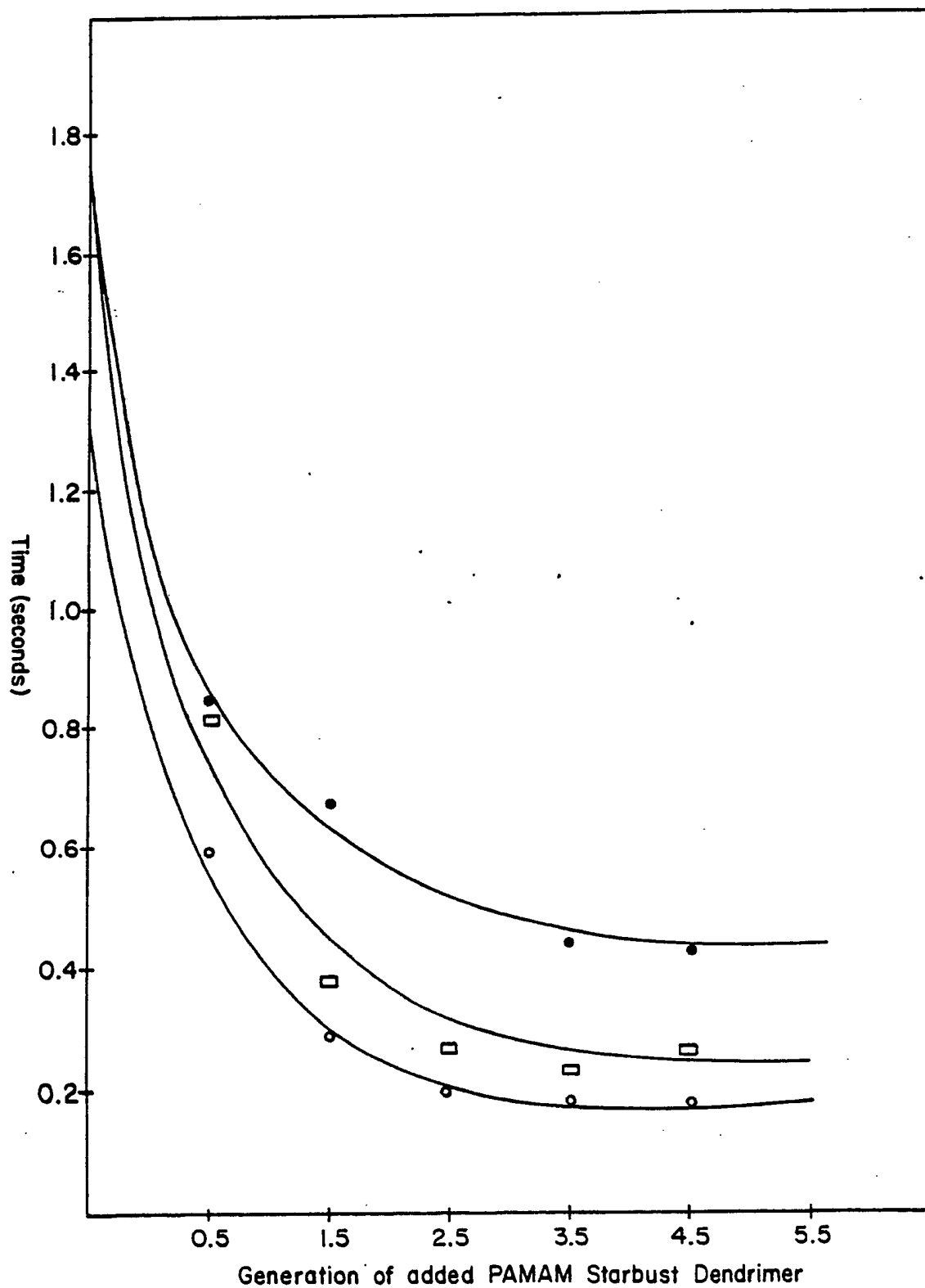
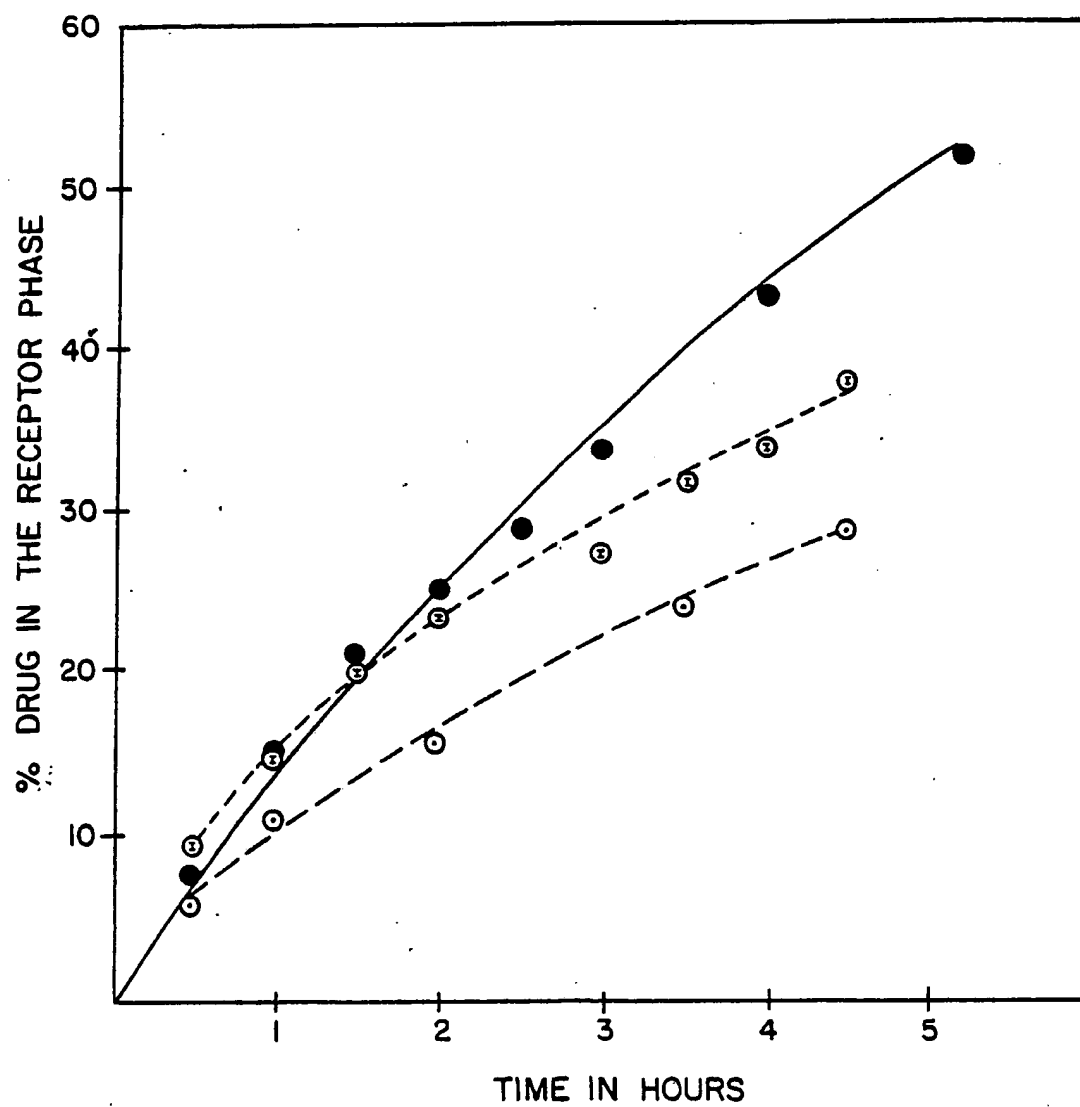


FIG. 4



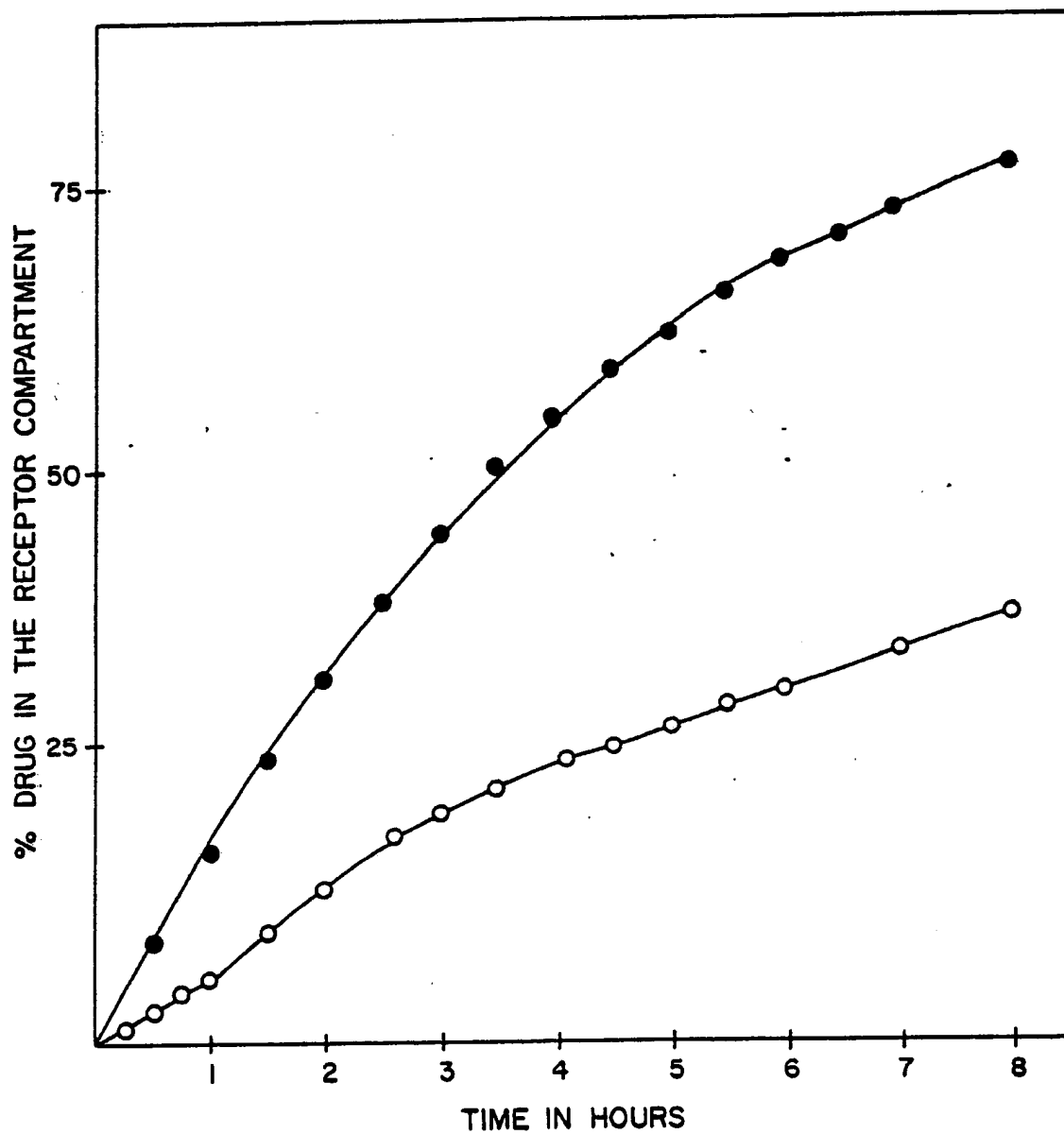
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FIG. 5



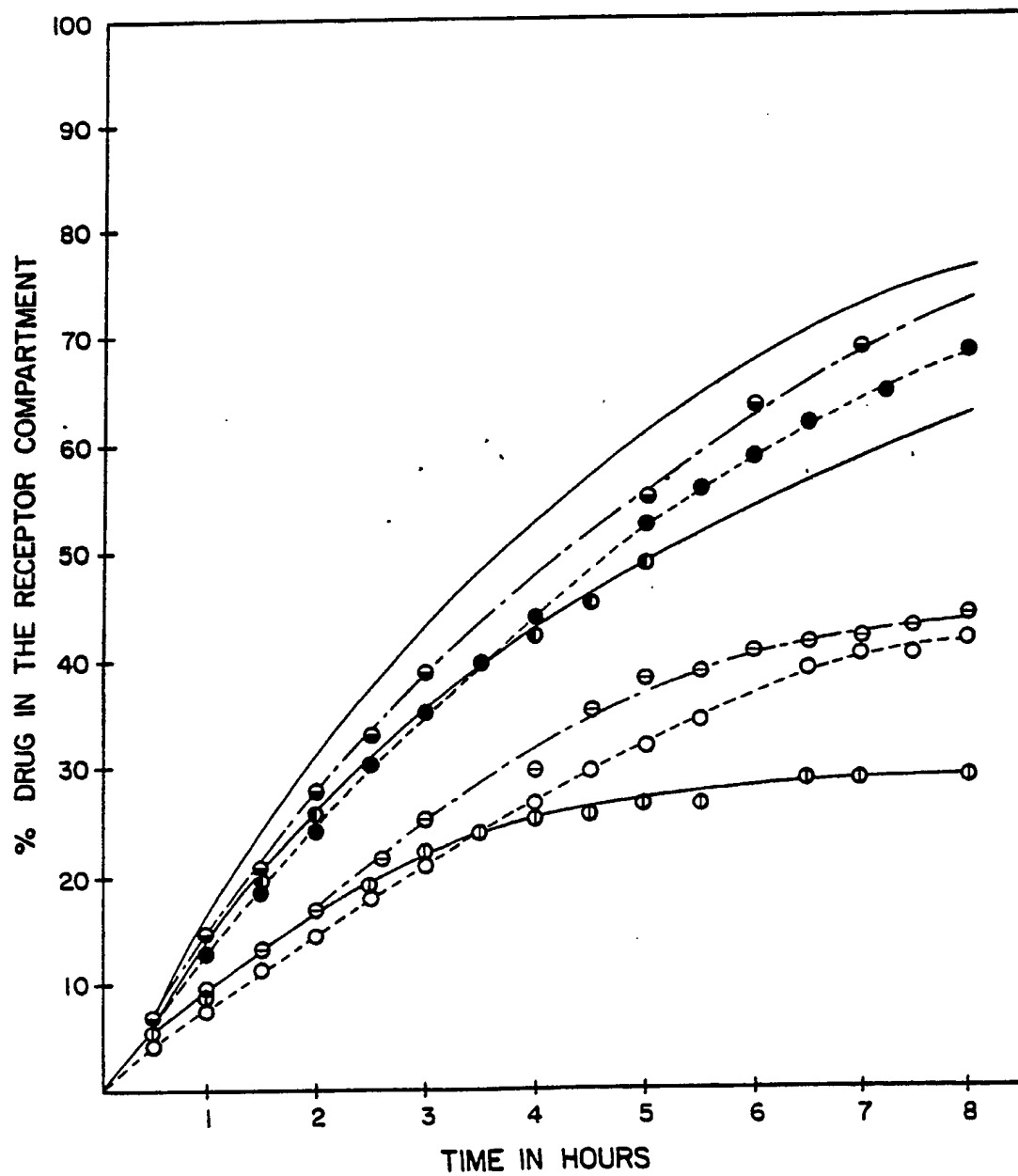
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FIG. 6



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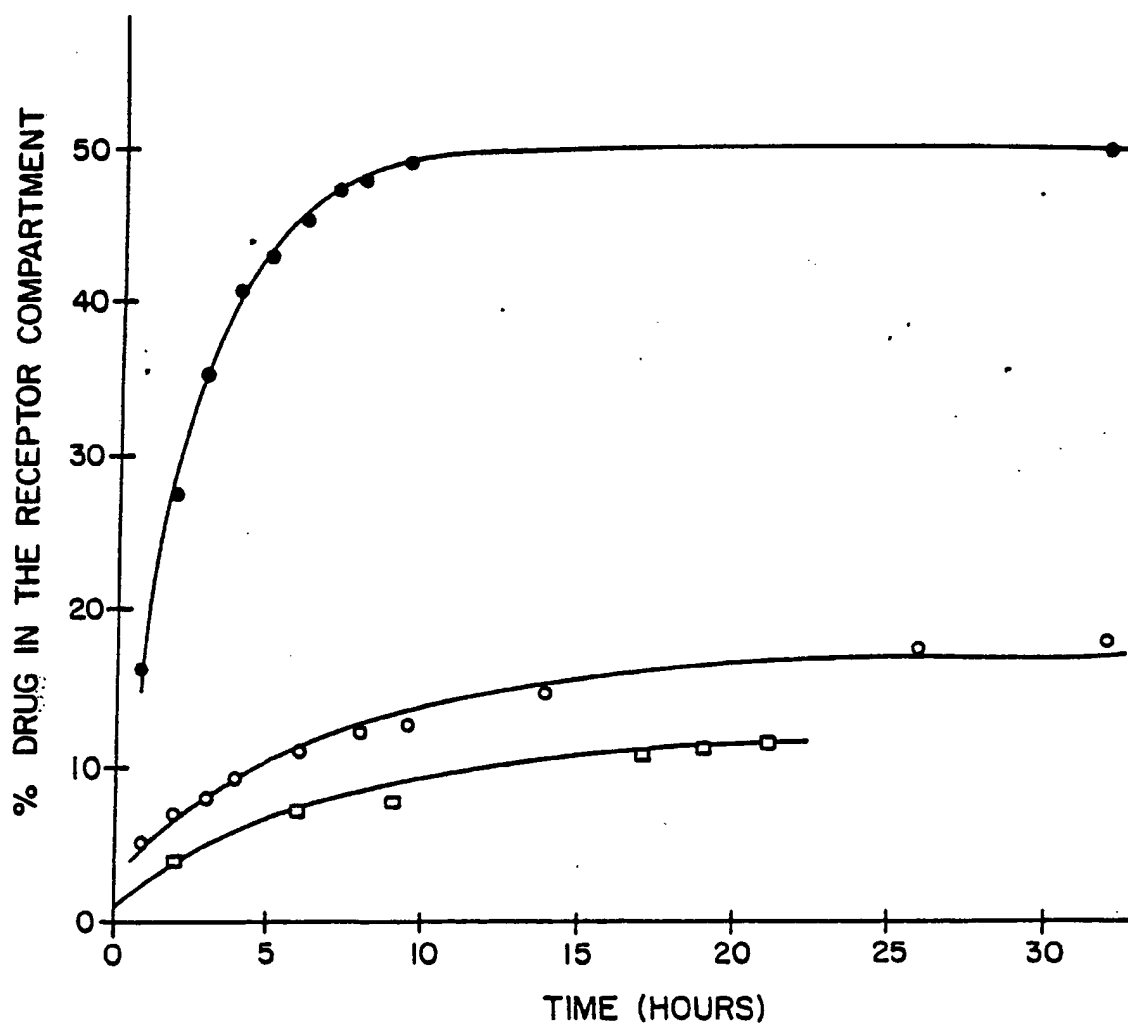
FIG. 7



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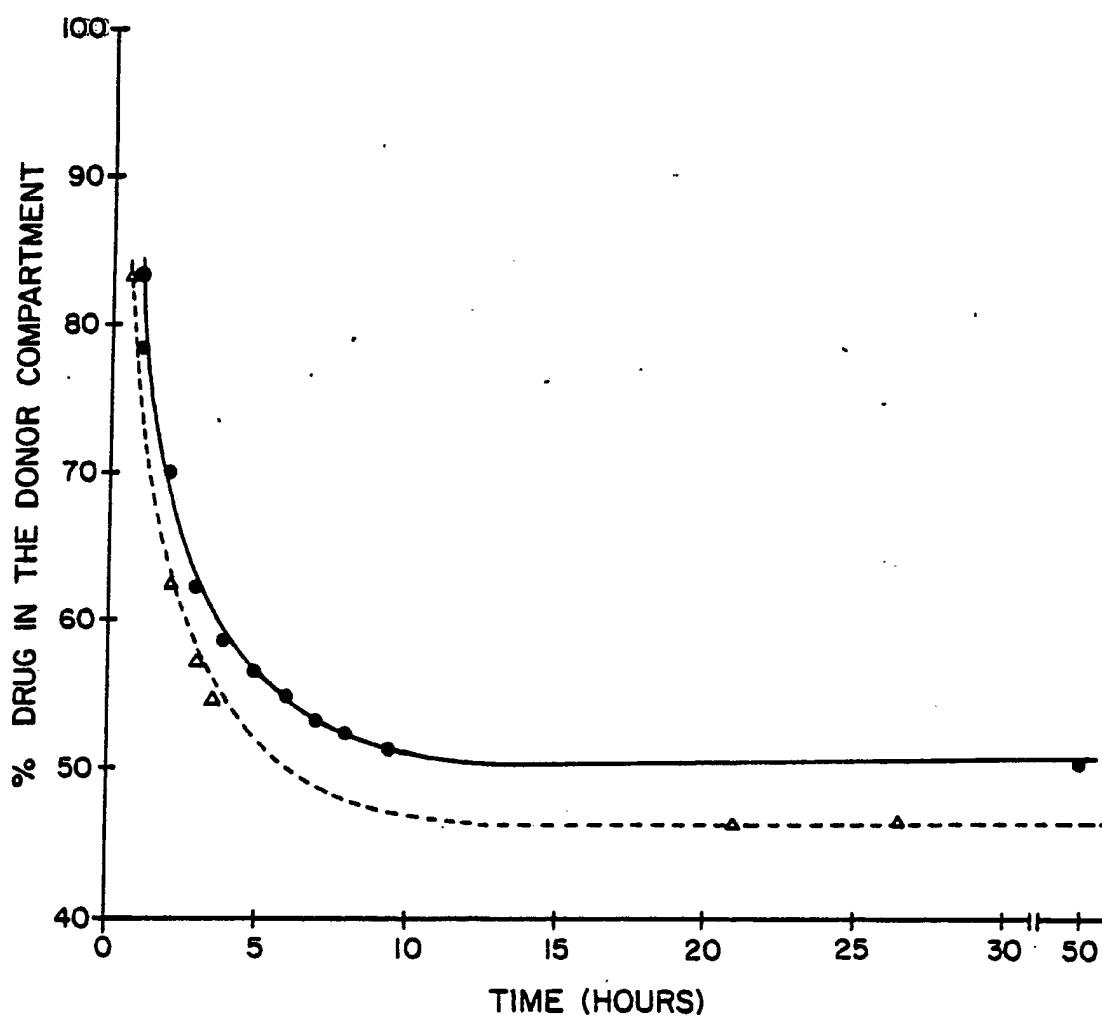
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FIG. 8



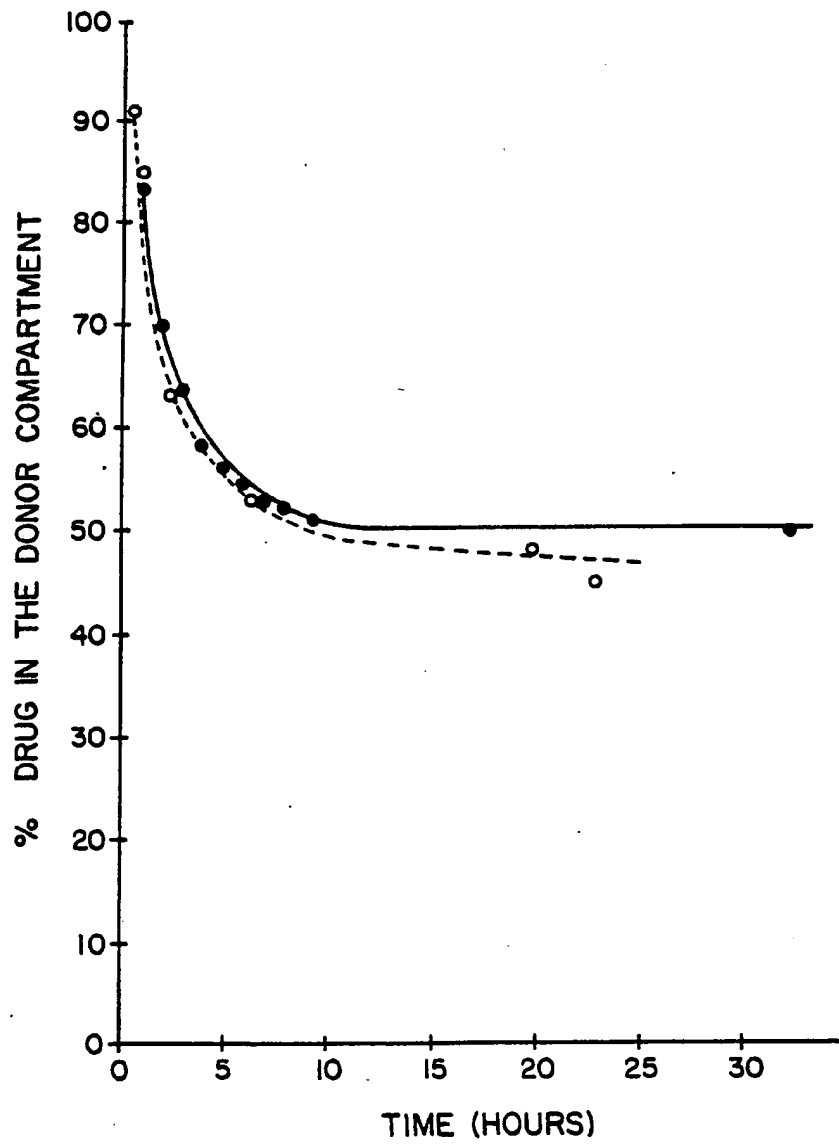
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FIG. 9



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FIG. 10



INTERNATIONAL SEARCH REPORT

International Application No PCT/US 87/02074

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC		
INT. CL. ⁴	A61K 49/02	
US. CL.	424/1.1	
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	424/1.1, 9 525/410, 416, 418, 451 528/310, 332, 350, 363, 397	
Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
A	US, A, 4,558,120 PUBLISHED 10 DECEMBER 1985 TOMALIA ET AL	1-27
P, A	US, A, 4,606,907 PUBLISHED 19 AUGUST 1986 SIMON ET AL	1-27
T	US, A, 4,694,064 PUBLISHED 15 SEPTEMBER 1987 TOMALIA ET AL	1-27
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²	Date of Mailing of this International Search Report ³	
29 OCTOBER 1987	24 NOV 1987	
International Searching Authority ¹	Signature of Authorized Officer ²⁰	
ISA/US	John Marles	

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

- I. Claims 1-23 drawn to a starburst conjugate and process of preparing classified in class 424 subclass 1.1.
- II. Claims 24-27 drawn to a process of preparing a starburst conjugate classified in class 528 subclass 363.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.